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(54) Title: A NOVEL PROSTATE/COLON TUMOR SUPPRESSOR GENE LOCATED ON HUMAN CHROMOSOME 8

(57) Abstract

This invention provides a novel nucleic acid molecule encoding a prostate/colon tumor suppressor gene product. The means and methods for detecting mutations and/or loss of prostate/colon tumor suppressor gene are provided. Also included within the scope of this invention are methods of suppressing the neoplastic phenotype of cancer cells having a defect in the prostate/colon tumor suppressor gene product. The invention also includes the means and methods for treating the cancer by administering the prostate/colon tumor suppressor gene.

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A NOVEL PROSTATE/COLON TUMOR SUPPRESSOR GENE LOCATED ON HUMAN CHROMOSOME 8

This invention is a continuation-in-part of U.S. application serial no. 08/246,604, filed May 20, 1994.

This invention was made in part with Government support under Grant Nos. CA 60358 awarded from Department of Health and Human Services and Grant Nos. CA 58236 and CA 55231, awarded from the National Cancer Institute. The government has certain rights in this invention.

Throughout this application, publications are referred to by first author name and date of publication in parenthesis. The disclosures of these publications are hereby incorporated by reference into the present application to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

This invention is in the field of tumor suppressor genes (anti-oncogenes) and relates in general to products and methods for practicing broad-spectrum tumor suppressor gene therapy of various human cancers. In particular, the invention relates to methods for treating tumor cells by: (1) administering vectors comprising a nucleic acid sequence coding for the novel proteins referred to herein as prostate tumor suppressor gene products (PTSG products); or, (2) administering an

effective amount of a protein coded for by the nucleic acid sequence. The invention also relates to diagnosis of certain cancers such as prostate and colon cancer using the cloned nucleic acids of this invention.

Cancers and tumors are the second most prevalent cause of death in the United States, causing 547,000 deaths per year. One in three Americans will develop cancer, and one in five will die of cancer (Scientific American Medicine, part 12, I, 1, section dated 1987). While substantial progress has been made in identifying some of the likely environmental and hereditary causes of cancer, the statistics for the cancer death rate indicate a need for substantial improvement in the therapy for cancer and related diseases and disorders.

A number of so-called cancer genes, i.e., genes that have been implicated in the etiology of cancer, have been identified in connection with hereditary forms of cancer and in a large number of well-studied tumor cells. Study of cancer genes has helped provide some understanding of the process of tumorigenesis. While a great deal more remains to be learned about cancer genes, the known cancer genes serve as useful models for understanding tumorigenesis.

Cancer genes are broadly classified into "oncogenes" which, when activated, promote tumorigenesis, and "tumor suppressor genes" which, when damaged, fail to suppress tumorigenesis. While these classifications

provide a useful method for conceptualizing tumorigenesis, it is also possible that a particular gene may play differing roles depending upon the particular allelic form of that gene, its regulatory elements, the genetic background and the tissue environment in which it is operating.

One widely considered working hypothesis of cancer is as follows: (1) Most of all human cancers are genetic diseases and (2) they result from the expression and/or failure of expression of specific genes (i.e. mutant versions of normal cellular growth regulatory genes or viral or other foreign genes in mammalian cells that cause inappropriate, untimely, or ectopic expression of other classes of vital growth-regulatory genes.

A simplistic view of the biologic basis for neoplasia is that there are two major classes of cancer genes. The first class consists of mutated or otherwise aberrant alleles of normal cellular genes that are involved in the control of cellular growth or replication. These genes are the cellular protooncogenes. When mutated, they can encode new cellular functions that disrupt normal cellular growth and replication. The consequence of these changes is the production of dominantly expressed tumor phenotypes. In this model of dominantly expressed oncogenes, a view that has predominated since the emergence of the concept of the genetic and mutational basis for neoplasia, it is imagined that the persistence of a single wild-type allele is not sufficient to prevent neoplastic changes in

the developmental program or the growth properties of the cell. The genetic events responsible for the activation of these oncogenes therefore might be envisioned as "single-hit" events. The activation of tumorigenic activities of the *myc* oncogene in Burkitt lymphoma, the expression of *bcr-abl* chimeric gene product in patients with chronic myelogenous leukemia, the activation of the H-*ras* and K-*ras* oncogenes in other tumors represent some of the evidence for the involvement of such transforming oncogenes in clinical human cancer. An approach to genetic-based therapy for dominantly expressed neoplastic disease presumably would require specific shutdown or inactivation of expression of the responsible gene.

Tumor suppressor genes

A more recently discovered family of cancer-related genes are the so-called tumor-suppressor genes, sometimes referred to as antioncogenes, growth-suppressor, or cancer-suppressor genes. Recent research suggests strongly that it is loss-of-function mutations in this class of genes that is likely to be involved in the development of a high percentage of human cancers; more than a dozen good candidate human tumor-suppressor genes have been identified in several human cancers. The tumor suppressor genes involved in the pathogenesis of retinoblastoma (RB), breast, and other carcinomas (p53), Wilm's tumors (wt 1, 2) and colonic carcinoma (DCC) have been identified and cloned. Some aspects of their role in human tumorigenesis have been elucidated.

The retinoblastoma gene (RB) is the prototype tumor suppressor. The RB gene encodes a nuclear protein which is phosphorylated on both serine and threonine residues in a cell cycle dependent manner (Lee et al., Nature, 329:642-645 (1987); Buchkovich et al., Cell, 58:1097-105 (1989); Chen et al., Cell, 58:1193-1198 (1989); DeCaprio et al., Cell, 58:1085-1095 (1989)). The molecular mechanisms by which RB participates in these cellular activities has not been completely elucidated. A current model holds that RB interacts with many different cellular proteins and may execute its functions through these complexes. If the function of RB protein is to maintain cells at G0/G1 stage, RB must "corral" and inactivate other proteins which are active and essential for entering G1 progression (Lee et al., CSHSOB, LVI:211-217 (1991)). This "corral" hypothesis is consistent with recent observations that an important growth-enhancing transcriptional factor, E2F-1, is tightly regulated by Rb in a negative fashion (Helin et al., Cell, 70:337-350 (1992); Kaelin et al., Cell, 70:351-364 (1992); Shan et al., Mol. Cell. Biol., 12:5620-5631 (1992); Helin et al., Mol. Cell. Biol., 13:6501-6508 (1993); Shan et al., Mol. Cell. Biol., 14:229-309 (1994)). The instantly disclosed protein, PTSG, binds to the Rb protein and thus participation in the regulation of mitosis.

The familial breast cancer gene, BRCA-1, has been mapped at chromosome 17 q21-22 by linkage analysis. It is not clear whether this gene will behave as a tumor suppressor or dominant oncogene. However, the gene involved in human familial cancer syndrome such as Li-

Fraumeni syndrome, p53, apparently acts as the classical tumor suppressor; similarly, the loss of RB gene is associated with hereditary retinoblastoma (Knudson, 1993, supra).

Multiple Steps and Oncogenetic Cooperation

Between these two extreme pictures of transforming oncogenes and purely recessive tumor-suppressor genes lie a number of additional mechanisms apparently involved in the development of neoplastic changes characteristic of many human tumors. It has been assumed for many years that most human cancers are likely to result from multiple interactive genetic defects, none of which alone is sufficient but all of which are required for tumor development to occur. The true roles of both the cellular protooncogenes and the growth-regulating tumor-suppressor genes in neoplasia of mammalian cells are thought to represent a complex set of interactions between these two kinds of genes.

One current theory of carcinogenesis is that for some tumorous pathologies like adenocarcinoma of the prostate, oncogenesis occurs through the selection of several genetic changes, each modifying the expression or function of genes controlling cell growth or differentiation (Nowell, P.C., Science 194:23-28 (1976); Weinberg, R., Cancer Res. 49:3713-3721 (1989)). Even though adenocarcinoma of the prostate is ranked first in incidence and second in mortality among neoplasms in men (Coffey, D.S., Cancer 71:880-886 (1993)), little is known

of the molecular basis of this common disease. For example, genetic alterations in colon cancer have been extensively studied and a model has been proposed in which the activation of oncogenes and loss of function of tumor suppressor genes is correlated with progressive clinical and histopathological changes observed during colorectal carcinogenesis (Fearon, E.R. and Vogelstein, B., Cell 61:759-767 (1990)). Indeed, a similar process of progressive genetic changes has been suggested to occur in prostate cancer (Isaacs, W.B. and Carter, B.S., Cancer Surveys, vol. 11, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 15-24 (1991)) but the exact location and mechanism of underlying genetic alteration remains unknown.

Known cancer genes have been shown not to be primarily responsible for prostate cancer. For example, mutations of cancer genes such as ras oncogenes or the tumor suppressor gene p53 have been found in only a small fraction (<10%) of early prostatic tumors (Carter et al., Proc. Natl. Acad. Sci. U.S.A., 87:8751-8755 (1990); Gumerlock et al., Cancer Res. 51:1632-1637 (1991); Bookstein et al., Cancer Res. 53:3369-3373 (1993)); however, mutations of the latter have been detected in 20-25% of late-stage primary tumors, suggesting that the p53 gene can participate in one of several alternative pathways of prostate tumor progression (Bookstein et al., Cancer Res. 53:3369-3373 (1993)).

Karyotyping and allelotyping of tumor cells also has been used to try to find the genetic mechanisms

responsible for prostate cancer. Cytogenetic studies of short-term cultures of primary prostatic cancers have disclosed several consistent chromosomal aberrations such as deletion of chromosomes 1p, 7q, or 10q (Atkin, N.B. and Baker, M.C., Hum. Genet., 70:359-364 (1985); Gibas et al., Cancer Genet. Cytogenet. 16:301-304 (1985); Lundgren et al., Genes Chrom. Cancer, 4:16-24 (1992)), whereas studies of allelic loss have suggested a somewhat different set of frequently lost chromosomal regions. Carter et al., Proc. Natl. Acad. Sci. U.S.A. 87:8751-8755 (1990), first reported non-random losses of chromosomes 10q and 16q each in ~30% of 28 tumors, and Kunimi et al., Genomics 11:530-536 (1991), showed losses of these same regions as well as of the p arms of chromosomes 8 and 10 at rates exceeding 50% in their set of 18 tumors.

Allelic loss of chromosome 8p is detected in 65% of prostate carcinomas, the highest rate of any chromosome arm. These rates compare to those of allelic losses of Rb in retinoblastoma, 100% of which have Rb mutation, and suggest the inactivation of a tumor suppressor gene in 8p. Interestingly, karyotypic deletion of 8p has been noted in androgen-unresponsive sublines of cell line LNCaP. No previously cloned suppressor genes are located in 8p.

In the study of Bergerheim et al. (Bergerheim et al., Genes Chromosom. Cancer 3:215-220 (1991)), alleles of the NEFL locus on chromosome 8p12-p22 were lost from tumors in 7 out of 8 informative patients, and those of the lipoprotein lipase locus (8p22) were lost in

6 out of 7 patients. Alleles of the PLAT locus (8p12-q11) were retained in some tumors losing more distal 8p loci, implying that the putative suppressor locus is located on 8p distal to PLAT. The most distal marker, D8S7, was lost in 3 out of 6 tumors. The exceptionally high rates of allelic loss of LPL and NEFL, and the failure to observe allelic losses starting distal to these loci, further suggested that the suppressor locus may be relatively close to LPL or NEFL.

Thus, in order to effectively diagnose susceptibility to prostate cancer and related pathologies and other related cancers, and for treatment, the locale of a tumor suppressor gene responsible for these pathologies must be identified and located. This invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

This invention is based on the discovery of a nucleic acid molecule encoding a novel prostate/colon tumor suppressor gene product (PTSG protein) having tumor suppression capability. The nucleic acid molecule has been mapped to the p22 region of chromosome 8. The expression of PTSG product in normal prostate and colon tissue, and its loss from some cases of prostate and colon cancer, support its identification as a tumor suppressor gene. The newly disclosed full length cDNAs encode two novel 348 and 347 amino acid proteins. This invention establishes for the first time that

inactivation of PTSG or PTSG product is responsible for prostate adenocarcinoma, colon cancer and other related cancerous pathologies, as provided herein.

Diagnostic methods using the nucleic acid and PTSG are disclosed. In one embodiment, oligonucleotide fragments capable of hybridizing with the PTSG gene, and assays utilizing such fragments, are provided. These oligonucleotides can contain as few as 5 nucleotides, while those consisting of about 20 to about 30 oligonucleotides being preferred. These oligonucleotides may optionally be labelled with radioisotopes (such as tritium, ^{32}phosphorus and ^{35}sulfur), enzymes (e.g., alkaline phosphatase and horse radish peroxidase), fluorescent compounds (for example, fluorescein, Ethidium, terbium chelate) or chemiluminsecent compounds (such as the acridinium esters, isoluminol, and the like). These and other labels, such as the ones discussed in "Non-isotopic DNA Probe Techniques", L.J. Kricka, Ed., Academic Press, New York, 1992, (herein incorporated by reference,) can be used with the instant oligonucleotides. They may be used in DNA probe assays in conventional formats, such as Southern and northern blotting. Descriptions of such conventional formats can be found, for example, in "Nucleic Acid Hybridization - A Practical Approach", B.D. Hames and S.J. Higgins, Eds., IRL Press, Washington, D.C., 1985, herein incorporated by reference. Preferably these probes capable of hybridizing with the PTSG gene under stringent conditions. The oligonucleotides can also be used as primers in polymerase chain reaction techniques, as

techniques are described in, for example, "PCR Technology", H.A. Ehrlich, Ed., Stockton Press, New York, 1989, and similar references.

According to the diagnostic method of the present invention, loss of the wild-type PTSG is detected. The loss may be due to either deletional and/or point mutational events. The PTSG alleles which are not deleted can be screened for point mutations, such as missense, and frameshift mutations. Both of these types of mutations would lead to non-functional PTSG products. In addition, point mutational events may occur in regulatory regions, such as in the promoter of the PTSG leading to loss or diminution of expression of the PTSG mRNA.

In order to detect the loss of the PTSG wild-type in a tissue, it is helpful to isolate the tissue free from surrounding normal tissues. Means for enriching a tissue preparation for tumor cells are known in the art. For example, the tissue may be isolated from paraffin or cryostat sections. Cancer cells may also be separated from normal cells by flow cytometry. These as well as other techniques for separating tumor from normal cells are well known in the art. If the tumor tissue is highly contaminated with normal cells, detection of mutations is more difficult.

Detection of point mutations may be accomplished by molecular cloning of the PTSG allele (or alleles) present in the tumor tissue and sequencing that

allele(s) using techniques well known in the art. Alternatively, the polymerase chain reaction can be used to amplify PTSG sequences directly from a genomic DNA preparation from the tumor tissue. The DNA sequence of the amplified sequence can then be determined. The polymerase chain reaction itself is well known inthe art. See e.g., Saiki et al., Science, 239:487 (1988); U.S. Patent 4,683,203; and U.S. Patent 4,683,195.

Specific deletions of PTSG can also be detected. For example, restriction fragment length polymorphism (RFLP) probes for the PTSG or surrounding marker genes can be used to score loss of PTSG allele. Other techniques for detecting deletions, as are known in the art can be used.

Loss of wild-type PTSG may also be detected on the basis of the loss of a wild-type expression product of the PTSG. Such expression products include both the mRNA as well as the PTSG protein product itself. POint mutations may be detected by sequencing the mRNA directly or via molecular cloning of cDNA made from the mRNA. The sequence of the cloned cDNA can be determined using DNA sequencing techniques which are well known in the art. The cDNA can also be sequenced via the polymerase chain reaction (PCR) which will be discussed in more detail below.

Alternatively, mismatch detection can be used to detect point mutations in the PTSG or its mRNA product. While these techniques are less sensitive than

sequencing, they are simpler to perform on a large number of tumors. An example of a mismatch cleavage technique is the RNase protection method, which is described in detail in Winter et al., Proc. Natl. Acad. Sci. USA, 82:7575 (1985) and Meyers et al., Science 230:1242 (1985). In the practice of the present invention the method involves the use of a labeled RNA probe which is complementary to the human wild-type PTSG. The riboprobe and either mRNA or DNA isolated from the tumor tissue are annealed (hybridized) together and subsequently digested with the enzyme RNase A which is able to detect some mismatches in a duplex RNA structure. If a mismatch is detected by RNase A, it cleaves at the site of the mismatch. Thus, when the annealed RNA preparation is separated on an electrophoretic gel matrix, if a mismatch has been detected and cleaved by RNase A, an RNA product will be seen which is smaller than the full-length duplex RNA for the riboprobe and the PTSG mRNA or DNA. The riboprobe need not be the full length of the PTSG mRNA or gene but can be a segment of either. If the riboprobe comprises only a segment of the PTSG mRNA or gene it will be desirable to use a number of these probes to screen the whole mRNA sequence for mismatches.

In a similar fashion, DNA probes can be used to detect mismatches, through enzymatic or chemical cleavage. See, e.g., Cotton et al., Proc. Natl. Acad. Sci. USA, 85:4397 (1988); and Shenk et al., Proc. Natl. Acad. Sci. USA, 72:989 (1975). Alternatively, mismatches can be detected by shifts in the electrophoretic mobility of mismatched duplexes relative to matched duplexes.

See, e.g., Cariello, Human Genetics, 42:726 (1988). With either riboprobes or DNA probes, the cellular mRNA or DNA which might contain a mutation can be amplified using PCR (see below) before hybridization.

DNA sequences of the PTSG from the tumor tissue which have been amplified by use of polymerase chain reaction may also be screened using allele-specific probes. These probes are nucleic acid oligomers, each of which contains a region of the PTSG sequence DNA sequence harboring a known mutation. For example, one oligomer may be about 30 nucleotides in length, corresponding to a portion of the PTSG DNA sequence. At the position coding for the 175th codon of the oligomer encodes an alanine, rather than the wild-type codon valine. By use of a battery of such allele-specific probes, the PCR amplification products can be screened to identify the presence of a previously identified mutation in the PTSG. Hybridization of allele-specific probes with amplified PTSG sequences can be performed, for example, on a nylon filter. Hybridization to a particular probe indicates the presence of the same mutation in the tumor tissue as in the allele-specific probe.

The kit of the present invention is useful for determination of the nucleotide sequence of the PTSG using the polymerase chain reaction. The kit comprises a set of pairs of single stranded DNA primers which can be annealed to sequences within or surrounding the PTSG in order to prime amplifying DNA synthesis of the PTSG itself. The complete set allows synthesis of all of the

nucleotides of the PTSG coding sequences. The set of primers may or may not allow synthesis of both intron and exon sequences. However, it should allow synthesis of all exon sequences.

The present invention is also directed to the administration of wild-type PTSG tumor suppressor gene or protein to suppress, eradicate or reverse the neoplastic phenotype in established cancer cells having no endogenous wild-type PTSG protein. The wild-type PTSG gene can be used to suppress or reverse the neoplastic phenotype or properties of established human cancer cells lacking wild-type PTSG protein. This suppression of the neoplastic phenotype in turn suppressed or eradicated the abnormal mass of such cancer cells, i.e. tumors, which in turn can reduce the burden of such tumors on the animal which in turn can increase the survival of the treated animals. The neoplastic properties which are monitored and reversed included the morphology, growth, and most significantly, the tumorigenicity of cancer cells lacking the normal PTSG protein. Thus, the "reduction of the burden of tumor cells" in an animal is a consequence of the "suppression of the neoplastic phenotype" following the administration of wild-type PTSG product tumor suppressor gene. "Neoplastic phenotype" is understood to refer to the phenotypic changes in cellular characteristics such as morphology, growth rate (e.g., doubling time), saturation density, soft agar colony formation, and tumorigenicity.

Therefore, the invention provides PTSG encoding vectors and PTSG proteins for use in treatment of tumors or cancers, and methods of preparing PTSG proteins and vectors suitable for use in methods of treatment. The invention also provides methods for assaying for molecules which bind to and effect PTSG.

The invention also provides methods of treatment for mammals such as humans, as well as methods of treating abnormally proliferating cells, such as cancer, such as prostate tumors and colon cancer or other tumor cells or suppressing the neoplastic phenotype. Broadly, the invention contemplates treating abnormally proliferating cells, or mammals having a disease characterized by abnormally proliferating cells by any suitable method known to permit a host cells compatible-PTSG encoding vector or a PTSG protein derivative to enter the cells to be treated so that suppression of one or more characteristics of the neoplastic phenotype or suppression of proliferation is achieved.

In one embodiment, the invention comprises a method of treating a disease characterized by abnormally proliferating cells, in a mammal, by administering an expression vector coding for PTSG to the mammal having a disease characterized by abnormal proliferating cells, inserting the expression vector into the abnormally proliferating cells, and expressing PTSG in the abnormally proliferating cells in an amount effective to suppress proliferation of those cells. The expression vector is inserted into the abnormally proliferating

cells by viral infection or transduction, liposome-mediated transfection, polybrene-mediated transfection, $CaPO_4$ mediated transfection and electroporation. The treatment is repeated as needed.

In another embodiment, the invention comprises a method of treating abnormally proliferating cells of a mammal by inserting a PTSG encoding expression vector into the abnormally proliferating cells and expressing PTSG product therein in amounts effective to suppress proliferation of those cells. The treatment is repeated as needed.

In another alternative embodiment, the invention provides a DNA molecule able to suppress growth of an abnormally proliferating cell. An example of a prostate/colon tumor suppressor protein is PTSG protein product having an amino acid sequence substantially according to SEQ ID NO. 1. In a more preferred embodiment, the DNA molecule has the DNA sequence of SEQ ID NO. 1, and is expressed by an expression vector. The expression vector may be any host cell-compatible vector. The vector is preferably selected from the group consisting of a retroviral vector, an adenoviral vector and a herpesviral vector. In another more preferred embodiment, the DNA molecule has the DNA sequence of SEQ. ID No. 2, and is expressed by an expression vector. The expression vector may be any host cell-compatible vector. The vector is preferably selected from the group consisting of a retroviral vector, an adenoviral vector and a herpes viral vector.

In another alternative embodiment, the invention provides a PTSG protein product having an amino acid sequence substantially according to SEQ ID NO. 2 and biologically active fragments thereof. In yet another alternative embodiment, the invention provides a PTSG protein having an amino acid sequence substantially according to Seq. ID No. 4 and biologically active fragments thereof.

In another alterative embodiment, the invention provides a method of producing a PTSG protein product by the steps of: inserting a compatible expression vector comprising a PTSG encoding gene into a host cell and causing the host cell to express PTSG protein.

In another alternative embodiment, the invention comprises a method of treating abnormally proliferating cells of a mammal ex vivo by the steps of: removing a tissue sample in need of treatment from a mammal, the tissue sample comprising abnormally proliferating cells; contacting the tissue sample in need of treatment with an effective dose of an PTSG encoding expression vector; expressing the PTSG in the abnormally proliferating cells in amounts effective to suppress proliferation of the abnormally proliferating cells. The treatment is repeated as necessary; and the treated tissue sample is returned to the original or another mammal. Preferably, the tissue treated ex vivo is blood or bone marrow tissue.

In another alternative embodiment, the invention comprises a method of treating a disease characterized by abnormal cellular proliferation in a mammal by a process comprising the steps of administering PTSG protein to a mammal having a disease characterized by abnormally proliferating cells, such that the PTSG protein is inserted into the abnormally proliferating cells in amounts effective to suppress abnormal proliferation of the cells. In a preferred embodiment, the PTSG protein fragments or derivatives thereof is liposome encapsulated for insertion into cells to be treated. The treatment is repeated as necessary.

BRIEF DESCRIPTION OF FIGURES

Figure 1 shows KSR2 (8p22) Southern analysis in human prostate cancer. Paired purified prostate cancer DNA (T) and noncancerous DNA (N) from the same patients. The 1.9-kilobase allele is lost in the tumor tissue of patient 4, the 3.3-kilobase allele is lost in the tumor tissues of patients 5 and 6. Patient 7 is not informative at this locus.

Figure 2 shows the percentage of prostate cancers with loss at loci studied on chromosome 8.

Figure 3 shows homozygous deletion of MSR in human prostate cancer. Primary tumor 23 has retained both alleles at D8S201, is uninformative at D8S163, has

lost the 6.3-kilobase allele at MSR, and is uninformative at D8S39. Metastatic tumor N2 has lost one allele at D8S201 and at D8S163, while demonstrating complete loss of sequences at MSR. Re-probing the same blot with the 15-65 probe for DCC (18q), a strong signal is obtained at 8 kilobases (kb), demonstrating the presence of high molecular weight DNA in the tumor lane. Both D8S39 alleles are present in tumor N2 and the intensity of the lower allele is multiplied 3-fold. Figure legend: bp, base pairs. For definition of T and N, see legend to Figure 1.

Figure 4 shows deletion map in human prostate cancer. Only tumors demonstrating chromosome 8p loss are illustrated. Samples 1-27 are primary tumors. Figure legend: N1 through N5 are metastatic prostate cancers. O, retained alleles, loss of heterozygosity, X, homozygous deletion.

Figure 5. Yeast artificial chromosome and radiation hybrid map of loci in chromosome band 8p22, a common region of allelic loss in multiple human cancers. Genomics 24:317-323.

Figure 6 shows homologous integration of the conversion vector, which results in amplification of a 1855 bp band.

Figure 7 shows the Southern blot of yeast DNA with radiolabeled hygro-gene probe that confirms the presence of the hygro^R gene in the YAC arm.

Figure 8. Long-range restriction map of YACs encompassing markers on chromosome band 8p22. DNA from YACs 946_c_9, 877_f_2, 932_e_9, and 766_a_12 embedded in agarose beads was digested with various rare-cutting restriction enzymes (A: Asc I, M: Mlu I; N: Not I; Nr: Nru I; Sf: Sfi I) and separated by PFGE as described in Methods. Southern blotting with selected cDNA (*italic*) and genomic DNA (roman) probes was performed to identify restriction fragments containing each probe (brackets). Probes found to be homozygously deleted in Tumor N2 (Figures 3 and 4) are shown in **bold**, and the deduced minimal (740 kb; thick line) and maximal (920 kb; thin line) extent of the deletion in this tumor is shown above. The N33 gene is located within the deletion as shown.

Figure 9. Nucleotide sequence and selected restriction sites of the insert of plasmid pBS-N33C(7), derived by cloning into pBluescript the 1.3 kb EcoRI-EcoRI insert from lambda phage clone λN33C (SEQ ID NO. 5), which was obtained by screening a human placenta cDNA library with selected cDNA probe N33. Selected restriction sites are shown. The first ~20 bp of sequence containing the Not I site are presumably artificially introduced during cDNA library construction.

Figure 10. Annotated double stranded sequence of N33 cDNA deduced from sequencing phage clone N33C(7) and RT-PCR clones A4 and A5. A 65-bp segment from nt 1186 to 1250 of N33C(7) and A4 sequence is absent from the A5 clone, so N33C(7) and A4 clones represent the

longer Form 1 whereas A5 represents the shorter Form 2 mRNA. The presumptive alternative splice results in the utilization of either of two translational stop sites as indicated. The predicted translational start site is also shown preceded by an in-frame stop codon (*).

Figure 11. ORF map for clone N33C(7) representing mRNA form 1. The longest ORF is nt 158 - 1202.

Figure 12. Translation of longest ORF from mRNA form 1 (SEQ ID NO. 1). The predicted 348 amino-acid polypeptide has MW 39674.13 daltons (SEQ ID NO. 3). The last 5 amino acids differ from the form 2 polypeptide.

Figure 13. ORF map for deduced mRNA form 2. The longest ORF is nt 158-1199.

Figure 14. Translation of longest ORF from mRNA form 2 (SEQ. ID NO 2). The predicted 347 amino-acid polypeptide has MW 39556.18 daltons (SEQ ID NO. 4). The last four amino acids diverge from the form 1 polypeptide.

Figure 15. Alignment of N33 form 1 and 2 polypeptides with hypothetical 37.7 kD protein encoded by ORF ZK686.3 from *C. elegans*. Four gaps were introduced into N33 to optimize alignment. 42% of residues were identical between human and *C. elegans* (underlined). The protein encoded by ORF ZK686.3 has MW 37.7 kD.

Figure 16. Northern blot of mRNA from normal human tissues (Clontech) hybridized with selected cDNA probes J2, J28 and N33. N33 mRNA is about 1.5 kb in size and is expressed in most tissues including heart, placenta, lung, liver, pancreas, prostate, testis, ovary and colon. Expression in spleen, thymus, small intestine and peripheral lymphocytes was low.

Figure 17. Northern blot of mRNA from human tumor cell lines hybridized with selected cDNA probes N33, P10, J2 and P16. Actin was used as a control for mRNA loading. N33 expression was not detected in 13 out of 14 colorectal carcinoma cell lines (SW480, SW837, SW1417, HT-29, SW403, LS174T, DLD-1, CACO-2, EB, SK-CO-1, RKO, HCT116 and COLO-302).

Figure 18. Northern blot of mRNA from tumor lines PPC-1, WI-38, H460, A549 (lanes 1 - 4), normal colonic mucosa (lane 5), and colon tumor lines SW837 and SW480 (lanes 6 and 7). N33 is expressed in mucosa dissected from colon.

Figure 19. RT-PCR assay for N33 expression in RNA from nine prostate cancer specimens (lanes 1-9). C: PCR control. N33 primers were N33GEX-f and -r. Primers for the p53, Rb, and G3PD genes were used as controls for RNA/cDNA quality. N33, Rb and p53 primers span exon boundaries and do not specifically amplify genomic DNA. Markedly decreased N33 expression was seen in cases 3, 6 and 9. In tissues expressing N33, both the upper (form 1) and lower (form 2) mRNAs can be seen.

Figure 20. Predicted sequence of N33 form 1 polypeptide. The conserved C-terminal 16 amino acids (boxed) was coupled to KLH and used to generate a rabbit polyclonal antibody.

Figure 21. Antibody recognition of an N33-glutathione-S-transferase fusion protein in *E. coli*. N33 RT-PCR products from placenta mRNA (primers N33GEX-f and -r) were cloned into pGEX-2T (Pharmacia). Clones A4 and A5 were isolated representing form 1 and form 2 mRNAs, respectively. Protein expression was induced by IPTG and cell lysates were separated by PAGE and transferred to membrane. The Western blot was incubated with affinity-purified polyclonal anti-N33 peptide antibody, and reactive bands were visualized by an alkaline-phosphatase conjugated secondary antibody and NBT/BCIP substrate. A fusion protein band of ~57 kD was detected in induced cells containing clone A4 but not A5 or other clones.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a novel gene encoding a protein referred to as PTSG protein. PTSG refers to two proteins: one composed of 348 amino acids and a second of 347 amino acids, each having a molecular weight of approximately 40kD.

As used herein, "nucleic acid" shall mean single and double stranded genomic DNA, cDNA, mRNA and cRNA. "Isolated" when used to describe the state of the nucleic acids, denotes the nucleic acids free of at least

a portion of the molecules associated with or occurring with the nucleic acid in its native environment.

Also provided by this invention is a recombinant expression vector or a recombinant replication vector comprising an isolated nucleic acid molecule corresponding to a tumor suppressor gene as well as host cells, e.g., bacterial cells, containing these vectors.

The treatment of human disease by gene transfer has now moved from the theoretical to the practical realm. The first human gene therapy trail was begun in September 1990 and involved transfer of the adenosine deaminase (ADA) gene into lymphocytes of a patient having an otherwise lethal defect in this enzyme, which produces immune deficiency. The results of this initial trial have been very encouraging and have helped to stimulate further clinical trials (Culver, K.W., Anderson, W.F., Blaese, R.M., Hum. Gene. Ther., 2:107 (1991)).

So far most of the approved gene transfer trials in human rely on retroviral vectors for gene transduction. Retroviral vectors in this context are retroviruses from which all viral genes have been removed or altered so that no viral proteins are made in cells infected with the vector. Viral replication functions are provided by the use of retrovirus 'packaging' cells that produce all of the viral proteins but that do not produce infectious virus. Introduction of the retroviral vector DNA into packaging cells results in production of

virions that carry vector RNA and can infect target cells, but no further virus spread occurs after infection. To distinguish this process from a natural virus infection. To distinguish this process from a natural virus infection where the virus continues to replicate and spread, the term transduction rather than infection is after used.

For the purpose of illustration only, a delivery system for insertion of a nucleic acid is a replication-incompetent retroviral vector. As used herein, the term "retroviral" includes, but is not limited to, a vector or delivery vehicle having the ability to selectively target and introduce the nucleic acid into dividing cells. As used herein, the terms "replication-incompetent" is defined as the inability to produce viral proteins, precluding spread of the vector in the infected host cell.

Another example of a replication-incompetent retroviral vector is LNL6 (Miller, A.D. et al., BioTechniques 7:980-990 (1989)), incorporated herein by reference. The methodology of using replication-incompetent retroviruses for retroviral-mediated gene transfer of gene markers is well established (Correll, P.H. et al., Proc. Natl. Acad. Sci. U.S.A. 86:8912 (1989); Bordignon, C. et al., Proc. Natl. Acad. Sci. U.S.A. 86:8912-8952 (1989); Culver, K. et al., Proc. Natl. Acad. Sci. U.S.A. 88:3155 (1991); Rill, D.R. et al., Blood 79(10):2694-2700 (1991)), each incorporated herein by reference. clinical investigations have shown

that there are few or no adverse effects associated with the viral vectors (Anderson, Science 256:808-813 (1992)).

The major advantages of retroviral vectors for gene therapy are the high efficiency of gene transfer into replicating cells, the precise integration of the transferred genes into cellular DNA, and the lack of further spread of the sequences after gene transduction (Miller, A.D., Nature, 357:455-460 (1992)).

The potential for production of replication-competent (helper) virus during the production of retroviral vectors remains a concern, although for practical purposes this problem has been solved. So far, all FDA-approved retroviral vectors have been made by using PA317 amphotropic retrovirus packaging cells (Miller, A.D., and Buttimore, C., Molec. Cell Biol., 6:2895-2902 (1986)). Use of vectors having little or no overlap with viral sequences in the PA317 cells eliminates helper virus production even by stringent assays that allow for amplification of such events (Lynch, C.M., and Miller, A.D., J. Virol., 65:3887-3890 (1991)). Other packaging cell lines are available. For example, cell lines designed for separating different retroviral coding regions onto different plasmids should reduce the possibility of helper virus production by recombination. Vectors produced by such packaging cell lines may also provide an efficient system for human gene therapy (Miller, A.D., Nature 357:455-460 (1992)).

Non-retroviral vectors have been considered for use in genetic therapy. One such alternative is the adenovirus (Rosenfeld, M.A., et al., Cell, 68:143-155 (1992); Jaffe, H.A. et al., Proc. Natl. Acad. Sci. USA, 89:6482-6486 (1992)). Major advantages of adenovirus vectors are their potential to carry large segments of DNA (36 kb genome), a very high titre (10^{11} ml^{-1}), ability to infecting tissues in situ, especially in the lung. The most striking use of this vector so far is to deliver a human cystic fibrosis transmembrane conductance regulator (CFTR) gene by intratracheal instillation to airway epithelium in cotton rats (Rosenfeld, M.A., et al., Cell, 63:143-155 (1992)). Similarly, herpes viruses may also prove valuable for human gene therapy (Wolfe, J.H., et al., Nature Genetics 1:379-384 (1992)). Of course, any other suitable viral vector may be used for genetic therapy with the present invention.

The other gene transfer method that has been approved by the FDA for use in humans is the transfer of plasmid DNA in liposomes directly to human cells in situ (Nabel, E.G., et al., Science, 249:1285-1288 (1990)). Plasmid DNA should be easy to certify for use in human gene therapy because, unlike retroviral vectors, it can be purified to homogeneity. In addition to liposome-mediated DNA transfer, several other physical DNA transfer methods such as those targeting the DNA to receptors on cells by complexing the plasmid DNA to proteins have shown promise in human gene therapy (Wu, G.Y., et al., J. Biol. Chem., 266:14338-14342 (1991);

Curiel, D.T., et al., Proc. Natl. Acad. Sci. USA, 88:8850-8854 (1991)).

The PTSG of the present invention may be placed by methods well know to the art into an expression vector such as a plasmid or viral expression vector. A plasmid expression vector may be introduced into a tumor cell by calcium phosphate transfection, liposome (for example, LIPOFECTIN)-mediated transfection, DEAE Dextran-mediated transfection, polybrene-mediated transfection, electroporation and any other method of introducing DNA into a cell.

A viral expression vector may be introduced into a target cell in an expressible form by infection or transduction. Such a viral vector includes, but is not limited to: a retrovirus, an adenovirus, a herpes virus and an avipox virus. When PTSG is expressed in any abnormally proliferating cell, the cell replication cycle is arrested, thereby resulting in senescence and cell death and ultimately, reduction in the mass of the abnormal tissue, i.e., the tumor or cancer. A vector able to introduce the gene construct into a target cell and able to express H-NUC therein in cell proliferation-suppressing amounts can be administered by any effective method.

For example, a physiologically appropriate solution containing an effective concentration of active vectors can be administered topically, intraocularly, parenterally, orally, intranasally, intravenously,

intramuscularly, subcutaneously or by any other effective means. In particular, the vector may be directly injected into a target cancer or tumor tissue by a needle in amounts effective to treat the tumor cells of the target tissue.

Alternatively, a cancer or tumor present in a body cavity such as in the eyes, gastrointestinal tract, genitourinary tract (e.g., the urinary bladder), pulmonary and bronchial system and the like can receive a physiologically appropriate composition (e.g., a solution such as a saline or phosphate buffer, a suspension, or an emulsion, which is sterile except for the vector) containing an effective concentration of active vectors via direct injection with a needle or via a catheter or other delivery tube placed into the cancer or tumor afflicted hollow organ. Any effective imaging device such as X-ray, sonogram, or fiberoptic visualization system may be used to locate the target tissue and guide the needle or catheter tube.

In another alternative, a physiologically appropriate solution containing an effective concentration of active vectors can be administered systemically into the blood circulation to treat a cancer or tumor which cannot be directly reached or anatomically isolated.

In yet another alternative, target tumor or cancer cells can be treated by introducing PTSG protein into the cells by any known method. For example,

liposomes are artificial membrane vesicles that are available to deliver drugs, proteins and plasmid vectors both in vitro or in vivo (Mannino, R.J., et al., Biotechniques, 6:682-690 (1988)) into target cells (Newton, A.C. and Huestis, W.H., Biochemistry, 27:4655-4659 (1988); Tanswell, A.K. et al., Biochimica et Biophysica Acta 1044:269-274 (1990)); and Ceccoll, J. et al., Journal of Investigative Dermatology, 93:190-194 (1989)). Thus, PTSG protein can be encapsulated at high efficiency with liposome vesicles and delivered into mammalian cells in vitro or in vivo.

Liposome-encapsulated PTSG protein may be administered topically, intraocularly, parenterally, intranasally, intratracheally, intrabronchially, means at a dose efficacious to treat the abnormally proliferating cells of the target tissue. The liposomes may be administered in any physiologically appropriate composition containing an effective concentration of encapsulated PTSG protein.

"Host-vector system" refers to host cells which have been transfected with vectors constructed using recombinant DNA techniques. Insertion of the vector or DNA can be accomplished by microcell transfer, retrovirus-mediated gene transfer, transfection, cell fusion, etc. The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms. Additionally, this invention provides a method of transforming a cell by

contacting the cell with the vector or DNA of this invention, under suitable conditions.

Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques, encompassed by the present invention. See, for example, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1989) and the various references cited therein. This reference and the cited publications are expressly incorporated by reference into this specification.

In addition, recombinant DNA methods currently used by those skilled in the art include the polymerase chain reaction (PCR) which, combined with the synthesis of oligonucleotides, allows easy reproduction of DNA sequences. A DNA segment of up to approximately 6000 base pairs in length can be amplified exponentially starting from as little as a single gene copy by means of PCR. In this technique, a denatured DNA sample is incubated with two oligonucleotide primers that direct the DNA polymerase-dependent synthesis of new complementary strands. Multiple cycles of synthesis each afford an approximate doubling of the amount of target sequence. Each cycle is controlled by varying the temperature to permit denaturation of the DNA strands, annealing the primers, and synthesizing new DNA strands. The use of a thermostable DNA polymerase eliminates the necessity of adding new enzyme for each cycle, thus permitting fully automated DNA amplification. Twenty-

five amplification cycles increase the amount of target sequence by approximately 10^6-fold. The PCR technology is the subject matter of United States Patent Nos. 4,683,195, 4,800,159, 4,754,065, and 4,683,202.

It is understood that limited modifications can be made to the primary sequence of the tumor suppressor gene of this invention without destroying its biological function, and that only a portion of the entire primary structure may be required in order to effect activity. It is further understood that minor modifications of primary amino acid sequence may result in proteins which have substantially equivalent or enhanced function as compared to the molecule within the vector pBS-N33c(7). These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental such as through mutation in hosts. All of these modifications are included as long as tumor suppressor function is retained. Other unique nucleic acid fragments of at least 10 nucleotides are useful as hybridization probes. The probes are useful to detect the predisposition to a cancer caused by the malfunction of this gene. The isolated nucleic acid fragments also are useful to generate novel peptides. These peptides, in turn, are useful as immunogens for the generation of polyclonal and monoclonal antibodies useful in diagnostic methods outlined below. Methods of preparing and using the probes and immunogens are well known in the art, and are briefly described below.

Also included within the scope of this invention are nucleic acid molecules that hybridize under stringent conditions to an isolated nucleic acid molecule encoding this tumor suppressor protein. Such hybridizing nucleic acid molecules or probes, can by prepared, for example, by random priming of this nucleic acid molecule. For methodology for the preparation of such fragments, see Sambrook et al. (Sambrook et al., "Molecular cloning: a laboratory manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 1.98-1.104 (1989).

Purified tumor suppressor polypeptide or protein also is provided by this invention. These polypeptides and/or proteins are useful to prepare antibodies, which in turn are useful for diagnosis. They can be produced by recombinantly expressing an isolated nucleic acid molecule of this invention using well known molecular biology techniques.

"Purified", when used to describe the state of the protein, polypeptide, or antibody, denotes such protein free of a portion of the other proteins and molecules normally associated with or occurring with the tumor suppressor polypeptide, protein or antibody in its native environment. As used herein the term "native" refers to the form of a protein, polypeptide, antibody or a fragment of thereof that is isolated from nature or that which is without an intentional amino acid substitution.

As used herein, the term "antibody" or "immunoglobulin" refers to a protein that is produced in response to immunization with an antigen and specifically reacts with the antigen. This includes polyclonal as well as monoclonal antibodies. Human and mammalian, for example, mouse, rat, rabbit and goat, are intended to be included in this definition. The most predominant human antibody produced is of the IgG isotype, having two light and two heavy chains linked by disulfide bonds, which constitute about 80% of total serum antibodies.

Anti-tumor suppressor antibodies can be generated as follows. Fragments of the DNA insert in pBS-N33c(7) were fused with glutathiones S-transferase protein. The fusion proteins are then expressed in E. coli. Transfused E. coli cells are grown in LB medium plus ampicillin. The culture mixture was diluted from 1:10 to 1:150, preferably 1:100, with LB medium and ampicillin added. The procedure for recombinant plasmid construction is described in Sambrook et al. (Sambrook et al., "Molecular cloning: a laboratory manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 1.98-1.104 (1989)). The fusion of the fragments into vector frames at the site of restriction enzymes is described in Proc. Natl. Acad. Sci. 83:4685-4689 (1986).

Using the above described procedure for fusing GST with PTSG DNA fragment, quantities of the fusion protein were prepared and purified by preparative SDS polyacrylamide gel electrophoresis according to procedure described in Sambrook et al. (Sambrook et al.,

"Molecular cloning: a laboratory manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 1.98-1.104 (1989)) and Harlow and Lane (Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988)). The fusion protein is eluted by overnight extraction and SDS. Soluble acrylamide can be removed by dialysis. The proteins are then concentrated. Purified fusion protein is useful as an antigen in generating specific anti-PTSG antibody.

Rabbits can be repeatedly injected, preferably at 14 day intervals with 1-20 μg, preferably 10 μg, of purified fusion protein mixed with complete Freund's adjuvant (initial injection) and then given booster injections of the same amount of the fusion protein in incomplete Freund's adjuvant (repeated injections). Complete Freund's adjuvant generally consists of an emulsion of the antigen, in this case the fusion protein, in saline and a mixture of an emulsifying agent, such as for example Arlacel A, in mineral oil with killed mycobacteria. Incomplete Freund's adjuvant is the same except that it does not have the mycobacteria.

The injections are repeated until sufficiently high titer of anti-fusion protein is detected, approximately for two months, to react with both GST and the fusion protein. To enrich for antibodies recognizing only prostate tumor protein determinants, two or more affinity columns can be prepared using a method generally described in Harlow and Lane (Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor

Laboratory, Cold Spring Harbor, NY (1988)). At least one column is coupled with glutathione S transferase (GST) protein and at least one column is loaded with the fusion protein. Both columns are appropriately precycled. Antibody is passed first through the fusion protein-Sepharose column and eluted with glycine buffer of pH 2.3. The eluate is neutralized and passed through the GST column several times to remove antibody specifically directed against GST. The purified anti-prostate tumor suppressor protein is useful for immunoprecipitation or immunostaining, for localization of prostate tumor suppressor protein and will be equally useful for diagnostic identification of PTSG in mammalian and human tissue samples. Thus, the purified proteins also are within the scope of this invention. It can be labeled with a detectable marker such as radioisotypes, dyes, enzymes and biotin.

The above methods can be modified using any standard procedure as shown, for example, in Harlow and Lane (Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988)).

The fusion proteins also can be used to generate monoclonal antibodies. Thus, this invention provides a monoclonal antibody directed to an epitope on the prostate tumor suppressor protein or polypeptide. In one embodiment of this invention, the monoclonal antibody is a mouse monoclonal antibody. In another embodiment of

this invention, the monoclonal antibody is a human monoclonal antibody.

For the isolation of mouse monoclonal antibodies, eight week old mice can be injected interperitoneally with about 50 micrograms of a purified prostate tumor suppressor polypeptide (prepared as described above) in complete Freund's adjuvant 1:1 volume. Mice are then boosted, at monthly intervals, with the polypeptide, mixed with incomplete Freund's adjuvant, and bled through the tail vein. On days 4, 3 and 2 prior to fusion, mice are boosted intravenously with 50 micrograms of the polypeptide in saline. Splenocytes are fused with non-secreting myeloma cells according to procedures which have been described and are known to those of ordinary skill in the art to which this invention pertains. Some time later, approximately two weeks later, hybridoma supernatant are screened for binding activity against the prostate tumor polypeptide as described hereinafter. Positive clones are isolated and propagated.

In addition, this invention also provides the monoclonal antibody described hereinabove conjugated to a therapeutic agent. For the purposes of this invention, suitable therapeutic agents include, but are not limited to, a therapeutic agent selected from the group consisting of radioisotopes, toxins, toxoids, and chemotherapeutic agents. Also provided by this invention is the monoclonal antibody described hereinabove conjugated to a detectable marker. Suitable detectable

markers include, but are not limited to, enzymes, radioisotopes, dyes and biotin. This invention further provides monoclonal antibodies as described hereinabove conjugated to an imaging agent. Suitable imaging agents include, but are not limited to radioisotopes, such as ^{32}P, ^{35}S and ^{131}I.

Also provided by this invention are pharmaceutical compositions comprising the purified prostate tumor suppressor polypeptide or protein described hereinabove alone, or conjugated to any one of the following: a detectable marker, a therapeutic agent, or an imaging agent, as described hereinabove and a pharmaceutically acceptable carrier. Further provided are pharmaceutical compositions comprising the monoclonal antibody described hereinabove alone, or conjugated to any one of the following: a detectable marker, a therapeutic agent, or an imaging agent. As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as phosphate buffered saline solution, water, emulsions, such as an oil/water emulsion, and various types of wetting agents.

As used herein, "antibody" also encompasses fragments of antibodies. The antibody fragments retain at least some ability to selectively bind with its antigen. Also encompassed by this invention are antibody fragments that have been recombinantly or chemically synthesized that retain the ability to bind the antigen of the corresponding native antibody. The ability to

bind with an antigen or hapten is determined by antigen-binding assays known in the art such as antibody capture assays (See, for example, Harlow and Lane, (Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988)). Antibody fragments retaining some binding affinity include, but are not limited to: Fab (the fragment which contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion with the enzyme papain to yield an intact light chain and a portion of one heavy chain); Fab' (the fragment of an antibody molecule obtained by treating with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule); $(Fab')_2$, the fragment of the antibody that is obtained by treating with the enzyme pepsin without subsequent reduction; $F(ab')_2$ is a dimer of two Fab' fragments held together by two disulfide bonds; Fv and single chain antibodies (SCA). Also within the scope of this invention are CDR grafted and chimeric antibodies retaining the ability to bind prostate tumor suppressor protein.

As used herein the term "chimeric antibody" refers to an antibody in which the variable regions of antibodies derived from one species are combined with the constant regions of antibodies derived from a different species. Chimeric antibodies are constructed by recombinant DNA technology, and are described in Shaw et al., J. Immun. 138:4538 (1987), Sun, L.K. et al., Proc. Natl. Acad. Sci. USA 84:214-218 (1987); Neuberger, M.S.

et al., Nature 314:268 (1985), Boulianne, G.L. et al., Nature 312:643-646 (1984); and Morrison, S.L. et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984), for example.

As used herein the term "CDR grafted" antibody refers to an antibody having an amino acid sequence in which at least parts of one or more CDR sequences in the light and/or variable domain have been replaced by analogous parts of CDR sequences from an antibody having a different binding specificity for a given hapten or antigen. The analogous CDR sequences are said to be "grafted" onto the substrate or recipient antibody (see European Patent Publication No. 0 239 400). The "donor" antibody is the antibody providing the CDR sequence, and the antibody receiving the substituted sequences is the "substrate" antibody.

A method of detecting the presence or absence, in a sample, of a protein, the absence of which is associated with a neoplasm, is provided by this invention. For detection of protein, the method will include cell staining with polyclonal or monoclonal antibodies raised against the protein. For example, this method comprises the steps of obtaining a suitable sample from a subject. Suitable samples include, but are not limited to: prostate tumor tissue, colon tumor tissue, lymph node tissue and bone marrow cells. The method requires contacting the sample with an agent specifically unique to the tumor suppressor protein under conditions favoring the formation of a complex with the agent then

detecting the presence of any complex formed. The absence of complex indicating the absence of a protein, which is associated with a neoplastic state such as prostate adenocarcinoma. Thus, this method is useful to diagnose prostate adenocarcinoma. For the purposes of this invention, suitable labeling agents are radioisotopes such as ^{32}P, ^{35}S and ^{131}I, but also includes, but is not limited to dyes and enzymes.

For use in this method, the agent can be an antibody raised against the protein or a unique subregion of the protein, the absence of which is associated with prostate cancer.

A method of detecting the presence or absence, in a sample, of a tumor suppressor gene or nucleic acid, the absence of which is associated with a neoplasm, is provided by this invention. This method comprises the steps of obtaining a suitable sample from a subject. Detection methods for the presence of nucleic acid in cells include hybridization of a nucleic acid probe with the nucleic acid of a cell. Such techniques are accomplished by methods well-known to those skilled in the art. See, for example, Sambrook et al. (Sambrook et al., "Molecular cloning: a laboratory manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 1.98-1.104 (1989)).

Suitable samples include, but are not limited to: prostate tumor tissue, colon tumor tissue, lymph node tissue and bone marrow cells. The method requires

contacting the sample with an agent specifically unique to wild-type or normal tumor suppressor gene under conditions favoring the formation of a complex with the agent then detecting the presence of any complex formed. The absence of complex indicating the absence of a wild-type gene, which is associated with a neoplastic state such as prostate adenocarcinoma. Thus, this method is useful to diagnose prostate adenocarcinoma. For the purposes of this invention, suitable detectable labels include radioisotopes e.g., ^{32}P, ^{35}S and ^{131}I, and includes, but not limited to additional labeling agents, such as dyes and enzymes. The agent can be a nucleic acid molecule corresponding to the tumor suppressor protein or a unique subregion thereof.

A kit for the detection, diagnosis or prognosis of prostate cancer is provided by this invention. The kit includes the reagents useful to carry out the methods described above and instructions for their use in the methods. A kit can be used for the direct genetic detection of pathological alterations in the prostate tumor suppressor gene, and can include oligonucleotides, primers for PCR analysis, reagents for SSCP, or sequencing, for example. The kits, reagents and methods also are useful for prognosis. For example, deletion may be indicative of a less favorable prognosis for recovery.

Also within the scope of this invention are compositions containing, at least, any of the above-references nucleic acids, peptides, or antibodies. These

compositions also can contain carriers or diluents such as phosphate buffered saline, emulsions or various wetting agents.

The following embodiments are intended to illustrate, not limit, the subject invention.

A. IDENTIFICATION OF ALLELIC LOSS

1. Tissue Samples

Prostate cancer tissue was obtained from patients undergoing radical prostatectomy for clinically localized prostate cancer between August 1988 and November 1994. None of the patients included in the study had been treated previously with chemotherapy or hormonal therapy. Prostate and seminal vesicle tissue was harvested and PTSG at -80°C as described in Bova, G.S. et al. (Bova, G.S. et al. "Homozygous deletion and frequent allelic loss of chromosome 8p22 loci in human prostate cancer." Cancer Res. 53:3969-3973.(1993)). Briefly, only clinically palpable tumors were eligible for the study, and only tumors palpable after surgical removal were harvested. The mean Gleason score (Meilinger, G.T. et al., (1967)) for the 42 primary tumors included in the study was 7.4±1.1 (SD) with a range of 5-9. Focal or established capsular penetration was seen in all 42 primary tumors studied, and thus all tumors studied fall into the T_3 category utilized in the recent tumor-nodes-metastasis classification of prostate cancer (Schroder, F.H. et al., (1992)). Histological

evidence of seminal vesicle invasion was seen in association with 16 of 42 (38%) of the primary tumors included in the study. Microscopic lymph node metastases were seen in 11 of 42 (26%) of the cases included in the study.

Harvested primary tumors were mounted and 6-μm sections were stained with hematoxylin and eosin. Forty-two primary prostate adenocarcinomas which could be trimmed to yield tissue containing greater than 70% tumor nuclei were selected for DNA analysis. Metastatic prostate adenocarcinoma tissue was available in ten cases from patients found to have palpable enlarged pelvic lymph nodes at the time of intended radical prostatectomy. A PTSG section taken at the time of surgery revealed metastatic adenocarcinoma and radical prostatectomy was not performed. Nodal tissue not needed for histological diagnosis was snap PTSG and -80°C and used for this study.

Paired noncancerous tissue (seminal vesicle, prostate, or blood lymphocytes) was obtained from each patient. Seminal vesicle or prostate tissue serving as source material for noncancerous DNA was examined every 300μm by PTSG section, and all tissue containing dysplastic or cancerous epithelia was rejected.

Preoperative serum prostate specific antigen levels were measured by monoclonal immunoradiometric assay (Hybritech, San Diego, CA).

2. DNA Preparation

Prostate specific antigen ("PSA") containing greater than 70% prostate cancer nuclei was isolated from surrounding tissue (containing benign prostate epithelia, stroma, lymphocytes, etc.) as much as possible using a cryostat sectioning technique described in Bos, J.L. et al., (1987). All prostate carcinomas studied were of the usual acinar type and were <2 cm in diameter. DNA isolation and quantification were performed as described in Carter, B.S. et al., (1990) and Burton, K., (1968).

3. Southern Analysis

Samples were cleaved with restriction endonucleases (BRL and New England Biolabs) with the buffers recommended by the supplier, using 10 units of enzyme/μg of DNA for *Msp*I digests and 7.5 units/μg for *Taq*I digests. Samples were electrophoresed in 0.8% agarose gels and transferred to Nytran nylon membranes (Schleicher & Schuell) in 0.4 M sodium hydroxide/0.6 M sodium chloride after depurination in 0.25 N HCl for 10 minutes. After covalent linking of the DNA to the membrane using UV irradiation (Stratagene), membranes were prehybridized in 10 ml 1 M NaCl/1% sodium dodecyl sulfate/10% Dextran sulfate at 65°C for 1 hour. DNA probes KSR2, NF 5.1, and MCT 128.2 were obtained from the American Type Culture Collection. Probes CI8-1, MSR-32 (MSR-macrophage scavenger receptor), CI8-319, and CI8-277 are cosmid probes that have been described in Emi, M. et

al., (1993). Probes were labeled using random hexamer priming and incorporation of [α-^{32}P]dCTP (Amersham) with the Klenow fragment of DNA polymerase I (Amersham). Probes CI8-1, MSR-32, CI8-319, and CI8-277 were boiled with sheared human placental DNA (Sigma), (0.2 mg/ml), cooled briefly on ice, and hybridized at 65°C overnight. Probes KSR2, NEFL, and MCT 128.2 were boiled with 0.5 ml of 2 mg/ml denatured sonicated salmon sperm DNA, briefly cooled on ice, and hybridized at 65°C overnight. After hybridization, membranes were washed in 0.1X standard saline-phosphate-EDTA 0.1% sodium dodecyl sulfate for 15 minutes and were subsequently exposed to Kodak XAR-5 film at -80°C in cassettes with amplifying screens.

Allelic loss was defined as the absence of one allele in prostate tumor DNA compared to the noncancerous paired control DNA. In some cases, when there was residual signal from contaminating normal tissue, densitometry was used for analysis. A sample was scored as having allelic loss if a 60% reduction was present in the diminished allele compared to its normalized retained counterpart.

Allelic multiplication using probe MCT 128.2 was defined as an increase in intensity of greater than 100% of one of two alleles present in tumor samples, or intensity differences of greater than 100% between tumor and normal alleles in homozygous cases when prior probing of the same blots demonstrated equal loading of DNA in tumor and normal lanes.

4. Microsatellite Analysis

Sequences for lipoprotein lipase ("LPL") (GZ 14) and Mfd 199 primer sets were as previously published in Tomfohrde, J. et al., (1992). One of each pair of primers (LPL GZ 14 and Mfd 199R) was end-labeled with [γ-^{32}P]ATP (ICN Biomedicals) using polynucleotide kinase (Boehringer-Manneheim) and 5X kinase buffer [0.25 M Tris, (pH 9.0), 50mM $MgCl_2$, 50mM dithiothreitol, and 0.25 mg/ml bovine serum albumin]. Six μl primer (10μM), 2.8μl 5X kinase buffer, 0.7μl kinase (9 units/μl), 1.5μl sterile deionized water, and 3.0 μl [γ-^{32}P]-ATP were combined and incubated at 37°C for 1 hour. Products were purified using G-25 spin columns (Boehringer-Mannheim). One μl labeled primer was added to 1 μl unlabeled primer (10 μM), 0.5 ml deoxynucleotide triphosphate mix (equal volumes of dATP, dCTP, dGTP, and dTTP each at 10mM), 5.5 μl sterile deionized water, and 10X Taq DNA polymerase buffer (Perkin-Elmer), 10 μl genomic DNA were added (2.5 ng/μl), and the mixture was heated to 94°C. After addition of Taq DNA polymerase solution (5 units), thermocycling was then performed with 30 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C (LPL) or 58°C (Mfd 199) for 30 seconds, and extension at 72°C for 30 seconds. This was followed by 72°C for 7 minutes. Products were then mixed with an equal volume of stop buffer containing 95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, and 20 mM EDTA. Samples were heat denatured at 94°C and 3-μl aliquots of each sample were loaded on 6% acrylamide gels containing 8.0 M urea. Gels were dried and exposed to Kodak XAR film. In

this study, allelic loss using microsatellite analysis was determined according to criteria similar to those used in Southern analysis described above.

5. Immunohistochemistry for MSR Protein

Sections of primary prostate cancer and adjacent noncancerous prostate (including areas of benign prostatic hypertrophy and normal prostate) were examined in five patients. Liver tissue from a single patient obtained at autopsy served as positive control for MSR staining. Well preserved central and peripheral zone prostate tissue was obtained from the same patient at autopsy and stained for MSR protein. This patient had no evidence of malignancy at autopsy and prostate tissue was normal on gross examination and histologically. Unfixed air-dried 6-μm frozen sections on glass slides were warmed to room temperature and fixed in 2% formaldehyde/10mM Tris, pH 7.4/150 mM NaCl/2mM $CaCl_2$ solution for 10 minutes and then incubated for 20 minutes at room temperature in 0.3% H_2O_2/absolute methanol solution. Slides were subsequently rinsed twice with 10mM Tris, pH 7.4/150 mM NaCl/2 mM $CaCl_2$ and then incubated at 37°C for 10 minutes in serum blocking solution (Zymed). Rabbit anti-human synthetic scavenger receptor peptide IgG (kindly provided by Dr. Tatsuhiko Kodama, University of Tokyo and is described in Kodama et al. (1988)) was then added (1:50) to each slide and incubated at 37°C for 30 minutes. The primary antibodies were detected with a biotinylated secondary antibody-streptavidin-peroxidase conjugate (Zymed).

6. Results

Fifty-two (52) prostate cancer specimens were examined for allelic loss using 8 polymorphic probes for the short arm of chromosome 8. Overall, 32 of 51 (63%) informative tumor specimens showed loss of at least one locus on chromosome 8p. The most frequently deleted region is observed at chromosome 8p22-21.2. Loss of one allele is identified in 14 of 23 (61%) tumors at D8S163 (12 of 19 primary tumors and 2 of 4 lymph node metastases) (Figure 1), in 15 of 32 (47%) tumors at LPL (15 of 30 primary tumors and 0 of 2 metastases), and in 20 of 29 (69%) tumors at MSR (17 of 26 primary tumors and 3 of 3 metastases), all on 8p22. Loss of one allele is identified in 16 of 27 (59%) tumors at D8S220 (12 of 22 primary tumors and 4 of 5 metastases) on 8p21.3-21.2 (Figure 2; Table 1).

In addition to loss of one allele at the MSR locus in a majority of tumors, one metastatic prostate cancer sample (N2) demonstrated homozygous deletion of MSR sequences. Hybridization of the same blot with the DCC probe 15-65 establishes the presence of intact DNA of equivalent or larger size in the N2 tumor lane (Figure 3). Repeat digestion of N2 DNA with MspI, TaqI, and EcoRI and probing for MSR has confirmed this finding, but at least one allele is present. The boundary of the homozygous deletion is thus delimited by D8S163 and LPL.

In contrast to 8p22-21.2, loci telomeric and centromeric to this region are largely retained, with

loss of one or more loci in only 9 of 48 (19%) of informative cases. Distal loci studied on 8p23 are largely retained, with loss in only 4 of 38 (11%) of informative cases at D8S140 and in only 3 of 22 (14%) of cases at D8S201 (Table 1). Loci studied on 8p11.2 and 8q24 are also infrequently deleted, with loss identified in 3 of 26 (12%) of informative cases at D8S194 and in 2 of 17 (12%) at D8S39.

Evidence of chromosome 8q multiplication was detected in 5 of 32 (16%) tumors probed at D8S39, including cases 4, 20, 21, N1, and N2 (Figure 3). Signals for one of two D8S39 (8q24) alleles were multiplied 2-3-fold after correction for DNA loading differences. All of the tumors with 8q amplification had loss of 8p in at least one locus.

Data from all primary and metastatic prostate cancers with demonstrated loss on chromosome 8p are summarized in Figure 4. Fifteen of 42 (36%) primary tumors studied and 5 of 10 (50%) metastatic tumors studied demonstrated retention of heterozygosity or were not informative at the 8 loci studied on chromosome 8p and these cases are not illustrated in Figure 4. All tumors with loss on 8p which are informative for MSR have lost at least one allele at this locus. Tumors 1, 18, 25, and N5 have retained D8S163 (KSR) but lost proximal loci including MSR. Tumors 24 and 25 have retained LPL but lost more distal loci including MSR. Those results confine the smallest region of overlap to the interval between D8S163 and LPL, flanking the MSR locus. Based on

the genetic map presented by Emi et al. (1993), this interval spans 14 cM in the male.

The observation of homozygous deletion at the MSR locus prompted us to perform a preliminary assessment of the macrophage scavenger receptor gene as a possible tumor suppressor gene. Prostate tissue was analyzed for expression of MSR protein using a highly specific polyclonal antibody as described by Kodama et al. (1988). Macrophage scavenger receptor protein was not detected among prostate cancer cells or noncancerous prostate epithelia. Scattered cells contained within the stroma of each of the prostate sections stained positively, consistent with staining in macrophages only.

To determine whether allelic loss on chromosome 8p correlates significantly with clinical parameters, preoperative serum PSA levels were reviewed, Gleason scored, and final pathological staging for each patient included in the study. Mean Gleason score did not differ between the two groups, with a mean of 7.3 in patients with 8p loss, and a mean of 7.6 in those with no 8p loss demonstrated. Preoperative PSA levels were available for 34 of 42 patients whose primary prostate cancer tissue was studied. Mean PSA level for the entire group of patients was 11.2 ng/ml (range 1.6-23.6). The mean preoperative PSA level for patients with 8p loss was 12.6 ng/ml, and for patients with no loss on chromosome 8p it was 9.3 ng/ml (analysis of variance, $P = 0.105$). Seminal vesicle invasion was observed in 11 of 27 (41%) patients with 8p loss and in 5 of 15 (33%) patients with no

seminal vesicle invasion (X^2, P = 0.055). Microscopic lymph node metastases were found in 9 of 27 (33%) of patients with 8p loss, and in 3 of 15 (20%) patients without 8p loss (X^2, P = 0.35). In summary, there is a trend toward higher preoperative PSA levels, more frequent lymph node involvement, and more frequent seminal vesicle involvement in patients with 8p loss demonstrated within their prostate cancers, but these trends are not statistically significant.

B. ISOLATION AND MAPPING OF 8p PROBES

1. Origin of Probes, Primers and Somatic Cell Hybrids

Plasmid probe pABL4-2 detecting D8S21 was obtained from R. White and its preparation is disclosed in Tsui, L.C. et al. (1989). Its insert was partially sequenced by priming from *E. coli* amber suppressor $tRNA^{Tyr}$ using oligonucleotide 5'-GAATCCTTCCCCCAC-3', and two PCR primers were designed to create an STS (Table 2). Lambda phage CRI-R191 detecting D8S26 was obtained from the ATCC. A 4.2 kb EcoRI restriction fragment of this phage was subcloned and partially sequenced, from which an STS was designed (Table 2). Cosmid CI8-487 detecting D8S233 was obtained from the Japanese Cancer Research Resources Bank. A 2.2 kb EcoRI restriction fragment of this cosmid was subcloned and partially sequenced to create an STS (Table 2). New STSs (Table 3) were created by partially sequencing random subclones of purified YAC DNA (see below). YAC end fragments (Table 3) were obtained by the

inverse PCR method of Albertsen and Thliveris (Joslyn et al., 1991). PCR products were ligated into TA cloning vector (Invitrogen) and sequenced, from which STSs were made (Table 3). The remaining primer sequences were obtained from sources indicated in Tables 2 and 3. DNA from a human chromosome 8 x CHO somatic cell hybrid mapping panel (Wagner et al., "A hybrid cell mapping panel for regional localization of probes to human chromosome 8." Genomics 10:114-125 (1991)) was kindly provided by M. Wagner.

2. Radiation Hybrids

A human x hamster hybrid line, GM10156b, containing human chromosome 8 as its only human component, was obtained from the NIGMS Mutant Cell Repository (Camden, NJ). The hybrid was exposed to 5000 rads of γ radiation and fused to the APRT- and HPRT-deficient Chinese hamster ovary cell line CHO-ATS-49tg by the method of Cox et al. (Cox et al., Science 250:245-250 (1990)). Following HAT selection, a total of 97 hybrid clones were obtained. The presence or absence of six marker loci (D8S26, MSR, D8S233, D8S261, D8S21 and LPL) in radiation hybrid DNA was determined by PCR with relevant primers listed in Table 2. Distances and orders among these markers were estimated using the Statistical Package for Radiation Hybrid Mapping (Cox et al., "Radiation hybrid mapping: a somatic cell genetic method for constructing high-resolution maps of mammalian chromosome." Science 250:245-250.). The TWOPOINT program was used to estimate recombination fractions and

retention frequencies. Trial maps were tested for support of order with the FOURPOINT program.

3. YAC Library Screening

A copy of the YAC library (Albertsen et al., Proc. Natl. Acad. Sci. U.S.A. 87:4256-4260 (1990)) was obtained from CEPH (Paris, France). The library was screened by PCR with ten loci listed in Table 2 by a heirarchical screening method (Green, E.D. and Olson, M.V. Proc. Natl. Acad. Sci. U.S.A. 87:1213-1217 (1990)). DNA pools were made from 4 plates, 8 rows and 12 columns; 58 superpools represented 384 clones each. Clones identified by plate/ row/ column address were streaked onto AHC-agar plates and confirmed by direct PCR of colonies or by PCR of yeast DNA (Ausubel et al., "Current Protocols in Molecular Biology." Greene Publishing Associates/J. Wiley & Sons, Inc., New York, NY. (1992)).

4. Embedding of Yeast DNA in Beads

YAC clone stocks were streaked onto AHC-agar plates. Single pink colonies were picked and grown in 5 ml of YPD media at 30°C overnight, then expanded to 100 ml for an additional 24 hours. Yeast cells were embedded in agarose beads by the method of Overhauser and Radic (Focus 9[3]:8-9, Bethesda Research Laboratories, Inc., Gaithersburg, Md., 1987) as follows: cells were recovered by centrifugation and washed twice in 20 ml of SE (75 mM NaCl, 25 mM Na_2EDTA, pH 8.0), then resuspended in 4 ml SE. Cell suspensions were transferred to 125 ml

Erlenmeyer flasks and warmed to 45°C. Genome-qualified low melting point agarose (1% in SE) and mineral oil were separately equilibrated to 45°C, and beakers containing 100 ml of ice-cold SE and a stir-bar were placed in ice buckets over magnetic stirrers at medium speed. Five ml of agarose were added to cells in each flask and mixed. Twenty ml of mineral oil were then added and the flask was swirled vigorously for 30 seconds to emulsify the contents, which were then poured immediately into an iced SE beaker. Beads were formed within 5 minutes. The mixture in each flask was transferred to several 50 ml centrifuge tubes and spun at low speed to separate aqueous and oil layers. Excess oil was removed and the contents respun. Residual oil, SE and floating beads were discarded and remaining beads (5-10 ml) were washed three more times with SE. The insides of tubes were wiped to remove trace oil and beads were pooled to one tube. Packed beads were resuspended in 1 volume of SE and cells were digested with 0.5 ml of 2-mercaptoethanol and 10 mg of freshly dissolved yeast lytic enzyme (70,000 U/g, ICN) per 10 ml final volume at 37°C for 2 hours. Beads were then spun as before, resuspended in 20 ml 1% (w/v) sarcosyl, 25 mM Na_2EDTA, pH 8.0, 50 ug/ml proteinase K, and incubated overnight at 50°C. The supernatant was removed and beads were washed in 20 ml TE with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) followed by two more washes in TE.

5. Preparation of YAC DNA by PFGE

A 0.6-cm thick, 1% agarose gel in 0.5X TBE was poured in a 20 cm wide x 14 cm long gel casting unit with 2 or 3 preparative wells. Wells were loaded with low-melt agarose beads containing YAC DNA and sealed with low-melt agarose. Yeast chromosomes were separated on a BioRad CHEF-DR III PFGE apparatus running at 60-120 sec switch times ramped over 24 hours at 6 V/cm at a 120° angle in 0.5X TBE at 14°C. The gel was stained in 1 μg/ml ethidium bromide in 0.5X TBE for 30 minutes and chromosomes visualized by UV irradiation. Slots 5-7 mm wide were cut parallel to and in front of each YAC to be isolated, and the gel was replace on its platform. Excess buffer and gel fragments were blotted away and slots were filled with 1% low-melt agarose (InCert, BioRad) in 0.5X TBE, which was allowed to set. The gel was replaced into the CHEF-DR III and equilibrated to 14°C. PFGE was run at a 180 seconds constant switch time for 4 hours. YAC bands were again visualized by UV illumination and cut out of the low-melt slot.

Gel slices were equilibrated with two changes of 1 X ß-agarase buffer (New England Biolabs [NEB]), the buffer was removed, and slices were melted at 65-70°C for 30 minutes. Melted slices were brought to 40°C and incubated for 1-2 hours with ß-agarase I (NEB) (5 U per gram of agarose), then chilled on ice and spun to remove undigested agarose. Supernatants were loaded onto Centricon 100 filter units (Amicon) with excess TE buffer and spun at 500 x g for 30 minutes to concentrate and

purify YAC DNAs. The resulting ~80 μl preps were further concentrated to 50 μl by Speed-Vac with recovery of supernatants as the final products.

YAC DNA (~50 ng) was digested with Bgl II and ligated into BamH I-digested pBluescript (Stratagene) by standard methods (Sambrook et al., "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 1.98-1.104 (1989)). The ligation mix was redigested with BamH I to reduce nonrecombinant background and transformed into *E. coli*DH10B (GIBCO-BRL) with X-gal and IPTG for blue-white selection per supplier's recommendations. Plasmids derived from white colonies were screened for use as single-copy probes in Hind III-digested human genomic DNA (Sambrook et al., "Molecular cloning: a laboratory manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 1.98-1.104 (1989)), then mapped on somatic cell hybrids and YAC clones as follows.

6. Biotin-Labelling of YAC DNA

Isolated YAC DNA (10 - 20 μl) was biotin-labelled by random primer extension in the presence of biotinylated dATP (BioPrime kit, BRL) in 50 μl volumes according to kit instructions. Successful labelling was verified by running 5 μl of reaction product on agarose gels and either visualizing a faint smear by ethidium bromide and UV irradiation or by transferring the DNA onto nylon membrane by standard methods. The membrane was blocked as for a Western blot and streptavidin-

conjugated alkaline phosphate was added directly without primary or secondary antibodies. The biotin-labelled DNA smear was visualized by the BCIP/NBT substrate reaction.

7. Oligonucleotides for Linkers and PCR

Two oligonucleotides were synthesized, 5'-CGATCTAGACCAGCACAATGG-3' (Primer 1) and 5'-CCATTGTGCTGGTCTAGATCGCACA-3' (Primer 2). Primer 2 was 5'-phosphorylated with ATP and T4 kinase (37°C, 30 min), heated to inactivate the enzyme, and annealed to equimolar amounts of Primer 1 to form a linker

5'-CGATCTAGACCAGCACAATGG-3'
3'-ACACGCTAGATCTGGTCGTGTTACC-P-5'
Xba I BstX I

that is blunt and phosphorylated on one end, and non-self-sticky on the other. DNA fragments flanked by these linkers are able to be PCR-amplified with Primer I.

8. Creation of Amplifiable Short-Fragment cDNA Libraries

RNA was isolated from tissues and cells using TriReagent (Molecular Research Center, Inc.) per manufacturer's instructions. Poly-A$^+$ RNA was selected from total RNA with biotinylated oligo-dT primers and streptavidin-conjugated paramagnetic particles (PolyATtract kit, Promega). Double-stranded cDNA was made from poly-A$^+$ RNAs and one sample of total RNA per

manufacturer's instructions with random primers and M-MLV RT (RiboClone cDNA synthesis kit, Promega). A final step with T4 DNA polymerase yielded blunt-ended cDNAs. cDNA made from total RNA was set aside for later use as a probe for ribosomal DNA (rDNA). Excess linkers (see above) were ligated to the poly-A$^+$-derived cDNAs with T4 DNA ligase. cDNA (1-5 μl) was amplified in 100 μl volumes using ~500 ng of Primer 1 and other PCR constituents at the usual concentrations. Conditions were (95°, 2.5') → (94°, 40"; 60°, 40"; 72°, 2.5') x 20 → (72°, 10'). PCR products were purified with Wizard PCR Prep spin columns (Promega) and eluted in 50 μl of 0.5 X TE. DNA was quantitated by DNA Dipstick (Invitrogen); typical yields were 500 ng of purified product per 100 ul reaction. Amplified cDNAs examined by agarose gel electrophoresis and ethidium bromide staining comprised a broad streak with maximal intensity at about 500 bp.

9. Blocking Repetitive Sequences in cDNA

Purified amplified cDNA (1-2 μg) was mixed with equal amounts (w/w) of Cot 1 DNA (GIBCO-BRL) and reaction volumes were adjusted to 80 μg/ml in 120 mM $NaPO_4$ buffer pH 7 (e.g., 50 μl of cDNA was reduced to 21 μl by SpeedVac, to which was added 1 μl Cot 1 DNA and 3 μl of 1 M $NaPO_4$ pH 7; the presence of TE was ignored). Reactions were overlaid with mineral oil and heated to 100°C for 10 minutes to denature, then incubated at 60°C for 20 hours (C_0t=20).

10. Hybridization of cDNA to YACs

The method of Morgan et al. (1992) was adapted with minor modifications. Biotin-labelled YAC DNAs (100 ng or ~10 μl of labelling reaction per hybridization) were heat-denatured and loaded into Centricon 100 filter units with blocked cDNAs (1 μg excluding Cot 1 DNA) and 2 ml of 1 mM $NaPO_4$, pH 7, and spun at 1000 x g for ~25 minutes. The phosphate buffer wash was repeated once, and the retentate (60-80 μl) was collected into microfuge tubes. Volumes were reduced to ~5 μl in the SpeedVac, at which point the hybridization mixes were adjusted to 120 mM $NaPO_4$ pH 7, 1 mM EDTA pH 8, and DNA concentrations (excluding Cot 1) of ~160 μg/ml (e.g., 1.1 μg in 7 μl). Reactions were overlaid with mineral oil, then incubated at 60°C for 60 hours (C_0t=120).

11. Capture, Amplification and Cloning of Selected cDNAs

Streptavidin-conjugated paramagnetic particles (Promega) were prewashed twice with TE + 1 M NaCl then incubated with completed hybridization reactions in 200 μl of TE + 1 M NaCl at room temperature for 15 minutes. Particles were collected magnetically and supernatants were removed. Particles were washed 5 times with 15 minutes incubations in 200 μl of 0.1 X SSC + 0.1% SDS, two at room temperature, then three at 60°C, with magnetic collection between each wash. Bound cDNA was eluted from particles with 100 μl of 50 mM NaOH for 15 minutes, neutralized with 100 μl of 1 M Tris-HCl pH 7.5,

and transferred to clean tubes. Supernatants were desalted and concentrated using NaI and silica matrices (Geneclean kit, Bio 101) per manufacturer's instructions into 20 μl volumes of TE. These cDNAs were re-amplified exactly as for the original libraries (see above) except that 5 μl of templates were used and PCR was carried out for 30 cycles. The resulting products were purified and blocked with Cot 1 DNA exactly as above. Selection with YAC DNA was also carried out a second time as above. Second-round selected cDNAs were captured as above and amplified one more time. Final PCR products were cloned directly into T-vector (Novagen), transformants of which were plated onto Tet + Amp LB-agar plates with X-gal and IPTG for blue-white selection per kit instructions.

12. Screening Recombinant Clones

White colonies (~75/selection) were picked with wooden toothpicks in duplicate onto two gridded Amp-agar plates, one having an overlaid circular nylon membrane and one without. A uniquely arrayed pattern of short streaks or dotted lines was created so that duplicate colonies on plates could be identified easily. After overnight growth at 37°C, the plain (master) plate was stored and the filter was lifted from the other plate and processed as for filter colony hybridization screening (Sambrook et al., "Molecular cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 1.98-1.104 (1989)) through 10% SDS, denaturation, neutralization, and 2 X SSC. Filters were baked, prewashed (pg. 1.101), prehybridized in 0.05 X

BLOTTO, then hybridized either sequentially or simultaneously with nick-translated total human DNA (Alu probe) and random-primed rDNA (ribosomal DNA probe). After washing and autoradiography, hybridization-negative colonies were picked from the master plate for further characterization. Mini-scale DNA preps were prepared and analyzed by BstX I or (HindIII + EcoRI) digestion and agarose gel electrophoresis. Inserts ranged in size from 250-500 bp and were excised from low-melt agarose gels and radiolabelled by random priming. Probes were hybridized to filters containing HindIII-digested DNA from YAC clones and to triplets of human, chromosome 8 human-mouse hybrid, and mouse genomic DNA to identify single-copy probes localized to human chromosome 8.

13. YAC content mapping

About 30 random subclones (Table 6) of YACs 932_e_9, 767_h_8, 802_f_11, 832_a_10, 821_f_7, and 885_c_8 were isolated and mapped to human chromosome 8 by Southern hybridization with human chromosome 8 x mouse hybrid cell line DNA or regional panel thereof (Wagner et al., Genomics 10:114-125 (1991)). These probes were mapped within the YAC contig by hybridization to Hind III-digested YAC DNA blots (note: Ele = E1). Three YAC end clones (YE766, YE843 and YE932) were isolated by inverse PCR as described above from YACs 766_a_12, 843_g_3 and 932_e_9 respectively and mapped to chromosome 8 and the YAC contig as above.

The CEPH megabase YAC library (~22,000 clones) was screened by PCR with primers for six simple tandem repeat polymorphisms (STRPs) and four RFLP-containing loci on several independent linkage maps (Fig. 5, Table 2). About 30 YAC clones were identified and confirmed with the initial screens. Additional clone addresses were obtained by searching AluPCR and fingerprinting overlap tables (Cohen et al., Science 250:245-250 (1993)). These clones were integrated into the YAC map after being tested for STS content. A set of 31 markers was used to assemble the map, including the ten screening STSs, one additional published STRP (D8S206), two expressed sequence tags (D8S294E and D8S297E) (Adams et al., Nature 355:632-634 (1992)), fifteen random YAC subclones and three YAC end clones (Table 3). PCR and Southern blotting methods were used in tandem to minimize the scoring of false positives and negatives. The YAC map was anchored to cytogenetic maps by the chromosomal location of the *MSR* and *LPL* (lipoprotein lipase) genes (8p22) (Mattei et al., Cytogenet. Cell Genet. 63:45-46 (1993); Emi et al., J. Biol. Chem. 268:2120-2125 (1993)) and by placing multiple probes in intervals A or B of the somatic cell hybrid panel described by Wagner et al. (Wagner et al., Genomics 10:114-125 (1991)) (Table 3), in which interval A is telomeric to interval B.

A single, large contig was formed from thirty-six YACs (Table 4, Figure 5). Parsimonious STS content mapping dictated a unique order for all ten of the original screening markers as tel - D8S26 - D8S511 -

D8S549 - *MSR* - D8S254 - D8S233 - D8S261 - D8S21 - *LPL* - D8S258 - cen. Cosmid CI8-245 (D8S335), which comprised a centromeric boundary for one or more allelic loss regions (Ohata et al., Genes Chromosom. Cancer 7:85-88 (1993); Emi et al., Genes Chromosom. Cancer 7:152-157 (1993); Fujiwara et al., Cancer Res. 53:1172-1174 (1994)), was not available from the Japanese Cancer Research Resources Bank and could not be incorporated into our map. It is tightly linked to and apparently centromeric of *LPL* (Emi et al., J. Biol. Chem. 268:2120-2125 (1993a); Emi et al., Genomics 15:530-534 (1993)). *CTSB* (cathepsin B), another RFLP marker which has been mapped to 8p22-p23.1 (Fong et al., Hum. Genet. 89:10-12. (1992)), was placed in hybrid interval A (MacGrogan et al., Genes Chromosom. Cancer 10:151-159 (1994)) and excluded from the physically mapped region D8S26 - D8S258 by its absence from this set of YAC clones (data not shown). Due to lack of sufficient YAC termini, we were unable to uniquely order some accessory markers such as D8S206, D8S294E, and D8S297E. As an incidental finding, probe E1, a random subclone of YAC 932_e_9, detected a Hind III RFLP in human DNA with two alleles, 12 kb and (8 kb + 4 kb).

YACs are subject to two kinds of rearrangement artifact, chimerism and internal deletion, which potentially can affect various aspects of physical mapping. Chimerism did not influence our STS content mapping and the derived order of loci because all markers were independently mapped to chromosome 8. Reinforcement against the effects of internal deletion was provided by the many interspersed accessory probes and by large

contig depth (redundancy). For example, a large internal deletion in YAC 767_h_8 encompassing probes E15, YE766, E1, E3, *MSR*, and E20 was postulated in order to retain the unity of at least six other YACs. On the other hand, this apparent deletion provided two additional "endpoints" with which to resolve the orders of two marker pairs.

Of the 97 radiation hybrids isolated, 17 retained one or more of the six genetic markers tested with retention frequencies for individual loci ranging from 0.12 to 0.17. At least one breakpoint was detected in 10 of 17 hybrids. Distance estimates between pairs of loci were generated by the TWOPOINT program (Table 4). The order of markers suggested by YAC mapping was tested by fourpoint analysis of the radiation hybrid data (Fig. 1). Calculated odds against inversion were greater than 1:1000 for all adjacent markers except D8S261 and D8S21, which were separated by only one breakpoint and a calculated theta value of 0.05, or 5 $cRay_{5000}$. Marker orders were therefore consistent among the genetic, YAC and radiation hybrid maps. The distance between D8S26 and *LPL* was ~9 cM on the genetic map and 90 cR_{5000} on the radiation hybrid map, suggesting a ratio of ~10 cR_{5000} per cM in this region.

B.14. Long range restriction mapping

Forty selected cDNA fragments (Table 7) have been isolated and mapped onto human chromosome 8 and the

YAC panel as above. Selection has been done with YACs 932_e_9, 802_f_11, 821_f_7, 877_f_2, and 946_c_9. A long-range restriction map of part of the 8p22 region was constructed (Figure 8). The map encompasses at least 25 probes from Tables 6 and 7. YAC DNA was digested with various rare-cutting restriction enzymes Asc I, Mlu I, Not I, Nru I, or Sfi I and separated by PFGE as described above. Southern blotting with was performed to identify restriction fragments containing each probe. The map was assembled based on standard mapping methodologies, including analysis of partial and double-enzyme digests. One important finding to note was that cosmid CI8-2644, obtained from Dr. Y. Nakamura, was located telomeric to the MSR gene rather than centromeric as suggested by Fujiwara et al. (Fujiwara et al., Genes Chromsom. Cancer 10:7-14 (1994)).

15. Mapping the homozygous deletion in Tumor N2.

DNA from the single metastatic prostate tumor with a homozygous deletion of MSR (Bova et al., Cancer Res. 53:3869-3873 (1993)) was examined by Southern blotting analysis with numerous newly isolated genomic and selected cDNA probes in order to map the extent of this deletion. Probes found to be completely deleted in this tumor (boldface, Figure 8) begin with MSR and extend telomerically through probes 877-15 and cCI8-2644. Markers E1c and 877-13 are the closest retained loci at the centromeric and telomeric ends, respectively. Based on the positions of lost and retained loci within mapped

restriction fragments (Figure 8), the minimum and maximum sizes of the homozygous deletion in this tumor were determined at 740 kb and 920 kb, respectively. The target tumor suppressor gene was presumptively located within this region and was inactivated by this deletion. N33 was located within this region (Figure 8).

The mapping of cCI8-2644 to a position near the telomeric deletion boundary was significant because it suggests that the common region of allelic loss detected in colorectal, liver, and lung cancers found by Fujiwara et al. (Fujiwara et al., Genes Chromosom. Cancer 10:7-14 (1994) overlaps extensively with this region of homozygous deletion. Thus any gene within the homozygous deletion may also be important in these other cancers. Furthermore, the size of the allelic loss region in the latter report must be larger than that stated in the paper (600 kb) and larger than the homozygous deletion in this tumor, ie., the homozygous deletion defines the smallest known critical region containing the putative tumor suppressor gene.

16. Sequence analysis of selected cDNA fragments

Sequencing of selected cDNA probes in Table 7 revealed the following: 1) P3 and P28 are identical to the 5' end of the MSR cDNA sequence, whereas P34 is derived from the 3' untranslated region of MSR. The isolation of fragments of known genes from the region indicated that the method cDNA selection was successful. 2) J28 overlaps P27, L3 and N28 and contains a partial

ORF encoding a novel predicted amino acid sequence with no close relatives in GENBANK or PIR. Other parts of this DNA sequence were nearly identical to those deposited in GENBANK by random cDNA sequencing. 3) J12 contains sequences 95% identical to that of human protein phosphatase type 2C alpha subunit, i.e., a known gene that has not yet been localized. The sequence differences were nonconservative and we suspected that J12 represented either a closely related gene or a pseudogene. We then cloned and sequenced the J12 locus at the genomic DNA level and found that it lacked introns and contained a single-base insertion that would destroy the conserved ORF. Thus we tentatively concluded that J12 was a pseudogene for human protein phosphatase type 2C alpha subunit. 4) L21, N21, N33, N36 and P14 overlap among each other and define a partial ORF with highly significant homology to a predicted gene in *C. elegans* identified by random sequencing of genomic cosmid or cDNA clones (SWISS-PROT P34669; GENBANK M88869, T01933, L17337; PIR S44911). The function of the *C. elegans* gene is unknown.

17. Cloning and sequencing of longer N33 cDNAs.

Based on preliminary expression data (see below), selected cDNA clone N33 was used as a probe to screen a placenta lambda phage cDNA library (Clontech). Clone λN33C was isolated and its 1.3 kb EcoRI-EcoRI insert was subcloned into pBluescript to yield pBS-N33C(7). Sequencing revealed a 1342-bp insert flanked by EcoRI sites (Figs. 9, 10) and encoding a long ORF (nt 158

- 1202) (Fig. 11). Oligonucleotide primers N33GEX-f and N33GEX-r were synthesized based on this sequence (Fig. 10) and used to amplify a segment N33 mRNA by RT-PCR of placenta mRNA. Two closely-spaced specific bands of ~950 bp were detected with an abundance ratio of roughly 1:2 (upper band: lower band). In order to further characterize these bands, RT-PCR products were cloned into pGEX-2T (Pharmacia) and two clones, A4 and A5, were isolated. Clone A4 was colinear with pBS-N33C(7) whereas A5 lacked nt 1186 - 1250 (65 bp) compared to the other clones. Consequently, we presume that N33C(7) and A4 clones represent the longer (Form 1) mRNA whereas A5 represents the shorter Form 2 mRNA. The ORFs encoded by the two forms differ over the last ~20 bp and utilize different termination codons (Figs. 10-14). The two ORFs are identical through residue 343 then encode 4 or 5 different C-terminal amino acids each.

One other sequence feature is that nt 1252 was C in N33C(7) but T in A4 and A5 (Fig. 10). This change does not affect the Form 1 ORF encoded by N33C(7) because it occurs after stop codon 1. It is not known whether this difference represents a natural polymorphism, a cloning artifact, or a mutation in one or more of these clones.

Both N33 predicted polypeptides were highly homologous ($p<e^{-100}$) to the *C. elegans* predicted cDNA ZK686.3. Alignment was optimized by introducing four gaps into N33, yielding ~42% identical residues between human and *C. elegans* gene (Fig. 15). Three 12- to 21-

residue subregions of N33 (e.g., ^{81}PRNYSMIVMFTALQP) retain >90% identity with ZK686.3, suggesting highly conserved functional motifs. On the other hand, the *C. elegans* gene lacks homologous residues of the first 35 amino acids of N33, and N33 internally lacks approximately 16 amino acids compared to ZK686.3 (Fig. 15), suggesting significant evolutionary divergence of the transcription units. N33 was not significantly related to any other sequences in GENBANK, PIR, SWISS-PROT or EMBL.

18. Expression of N33 in tissues, tumors and cultured cells.

Various selected cDNA clones were used to probe Northern blots containing mRNA from several normal human tissues, examples of which are shown in Fig. 16. A single mRNA of about 1.5 kb in size was detected with N33 probes in most tissues including heart, placenta, lung, liver, pancreas, prostate, testis, ovary and colon. Expression in spleen, thymus, small intestine and peripheral lymphocytes was low. Expression detected by another clone, J2, was seen mostly in skeletal muscle and testis, whereas two messages detected by clone J28 were found principally in placenta, testis and ovary. Expression of a tumor suppressor gene is expected in the tissues of origin of the target tumor types, so N33 but not J2 or J28 had expression patterns consistent with a suppressor gene for prostatic, colorectal and perhaps other cancers.

Northern analysis of mRNA from tumor cell lines showed expression of N33 in 3 of 3 prostate lines and 3 of 3 lung lines, but in only 1 out of 14 colorectal cancer cell lines (Fig. 17). In order to further determine the significance of this finding, the mucosa of a colon specimen (precursor tissue for colonic adenocarcinoma) was dissected from the colonic wall and tested for N33 mRNA, and specific expression was observed (Figure 18, lane 5).

Finally, small amounts of total RNA were extracted from nine fresh prostate cancer samples (7 primary tumors and 2 metastases). Cryomicrotome-directed dissection was employed to reduce the numbers of contaminating nonneoplastic cells in primary specimens, but some level (typically, ~20%) of infiltrating cells was unavoidable. Because of limiting amounts of available RNA, RT-PCR with N33-specific primers was employed to quantitate N33 expression. Primers from Rb, p53 and G3PD were used to control for RNA quality and cDNA synthesis. Markedly decreased expression of N33 was observed in three cases (lanes 3, 6 and 9), where lane 6 RNA was obtained from Tumor N2 to verify the function of this assay. Lane 3 and 9 RNAs were obtained from primary tumors, in which some quantity of N33 message is expected to be contributed by nonneoplastic cells. These findings together with the lack of expression in colorectal cell lines supported the identification of N33 as a candidate prostate and colorectal tumor suppressor gene.

19. Mechanism of loss of N33 expression in tumor cells and tissues.

The basis for the lack of N33 expression in colorectal tumor cells and prostate tumor tissues is unknown, but could be due to somatic mutations (e.g., affecting mRNA epxression or stability), methylation changes, or other epigenetic regulatory factors. Whereas one prostatic tumor is known to have a large homozygous deletion in band 8p22, the genetic status of additional primary and metastatic prostate and colorectal tumors are determined by several methods, as follows: 1) Southern blots of tumor DNAs are hybridized with N33 cDNA probes and other 8p22 markers to detect homozygous deletions or genetic rearrangements 2) the structure of the PTSG locus is determined by cloning / sequencing at the genomic DNA level by standard techniques. For example, a P1 clone containing the N33 gene has been isolated and is sequenced with primers from the cDNA sequence, revealing exon/intron boundaries and flanking intronic sequences. PCR primers for amplifying each exon is synthesized. Amplification and sequencing of tumor DNA is then performed to detect the presence of subtle small deletions or point mutations. 3) The presence of LOH is determined by comparing alleles at polymorphic markers in tumor vs. normal DNA from each patient. 4) Specific tests for DNA methylation is performed by comparing the Southern blot patterns of tumor DNAs digested with methylation-sensitive and -insensitive enzymes. For example, MspI- and HpaII-digested DNA is compared. The VHL gene, a tumor suppressor gene for renal cell

carcinoma, is known to be somatically inactivated by methylation in some cases (Herman et al., Proc. Natl. Acad. Sci. USA, 91: 9700-9704 (1994)).

20. Improved tools for detecting N33 inactivation.

Detection of N33 expression or lack thereof would be considerably simplified by immunohistochemical assays for the N33 polypeptides in tissue sections. Antibodies reactive to one form of N33 protein was made as follows: a conserved 16-amino acid peptide at the N33 C-terminus (Fig. 20) was coupled to KLH and used to immunize rabbits. After six weeks, serum was harvested and antibodies were affinity-purified against a peptide column. These polyclonal antibodies were tested in a Western blot of recombinant N33 fusion proteins expressed in *E. coli*. (Fig. 21). As described above, clones A4 and A5 (partial N33 proteins fused to the glutathione-S-transferase gene carried in expression vector pGEX-2T) were obtained representing form 1 and form 2 mRNAs, respectively. Protein expression was induced by IPTG and cell lysates were separated by PAGE and transferred to membrane. The Western blot was incubated with affinity-purified polyclonal anti-N33 peptide antibody, and reactive bands were visualized by an alkaline-phosphatase conjugated secondary antibody and NBT/BCIP substrate. A fusion protein band of ~57 kD was detected in induced cells containing clone A4 but not A5 or other clones.

C. YAC TRANSFER TO MAMMALIAN CELLS

1. Retrofitting YAC Clones With Hygromycin Resistance

The plasmid vector pLUSH containing segments of the telomeric end of the YAC4 vector, a bacterial Kan^R gene, the yeast Lys2 auxotrophy gene, and the mammalian $hygromycin^R$ gene (see map) was kindly provided by D. McElligott (Scripps Research Institute). pLUSH DNA was linearized by Sal I digestion and 5-10 μg was used to transform YAC-containing yeast cells using an alkali cation yeast transformation kit (Bio 101, Inc.) per manufacturer's instructions. Cells were plated on "triple drop-out" media ($trp^-ura^-lys^-$) to select for clones containing both the YAC and the conversion vector. Colonies were picked after 3-4 days and grown overnight in 2 ml of YPD medium. Yeast DNA was prepared and tested for homologous integration of pLUSH by PCR with primers: 5'-CTTGAGATCGGGCGTTCGACTCGC-3' and 5'-TGAACGGTGATCCCCACCGGAATTG-3' (Hermanson et al., Nucl. Acids Res. 19:4943-4948 (1991). Reactions were carried out in 20 μl volumes with 100 ng of each primer in standard buffers plus 10% DMSO. Reaction conditions were 95°C, 2.5 min, then 35 cycles of 95°C, 40 sec; 60°C, 40 sec; and 72°C, 2 min), followed by 72°C for 10 min. Homologous integration of the conversion vector results in amplification of a 1855 bp band (Figure 6). The presence of the $hygro^R$ gene was confirmed by Southern blotting of yeast DNA with a radiolabelled hygro gene probe (Figure 7).

2. Spheroplast Fusion and Selection of Transformants

A number of methods are available for transfer of YACs to mammalian cells. The spheroplast fusion protocol of Silverman et al., Mol. Cell. Biol. 13:5469-5478 (1993) was used. In brief, yeast cells grown by standard methods were pelleted, washed and resuspended in isotonic medium and cell walls digested with yeast lytic enzyme to produce yeast spheroplasts. These were layered on top of pelleted cultured mammalian cells such as NIH 3T3 cells or human tumor cells (50:1 numerical ratio) and incubated in the presence of polyethylene glycol 1500 (Boehringer-Mannheim) for 2 min at RT to induce fusion. Cells were diluted in tissue culture medium and incubated for 48 hr, after which selection with 300 μg/ml hygromycin was begun. Hygro-resistant colonies were apparent at approximately 3 weeks.

3. Genetic Analysis of Transformants

The presence of substantial portions of the YAC of interest was verified by PCR amplification or Southern blot detection of known genetic markers in the YAC (Table 8). For transfer of YACs to human cells, polymorphic markers were used such that allele sizes in the YAC differed from alleles already present in the parental cell. Retention of only part of transferred YACs can also be detected by these methods, and correlation of retained portions of YACs with phenotypic properties can be used to localize a tumor suppressor activity to a subregion of that covered by the YAC.

4. Phenotypic Analysis of Transformants

The phenotype of tumor cells after transfer of tumor suppressor genes can be assessed by a common set of assays regardless of whether transfer method, e.g., microcell transfer, retrovirus-mediated gene transfer, transfection, cell fusion, etc. Growth rate *in vitro* (^{3}H-thymidine incorporation), growth of transformants in soft agar, and tumorigenicity in nude mice can be compared in modified and parental cells to assess for tumor suppression activity, and thus, insertion of the vector and/or gene.

The preceding examples have been provided only to illustrate, not limit, this invention. It is understood that various modifications and additions can be made to this disclosure without departing from the spirit of this invention. Accordingly, this invention is defined by the following claims.

D. TESTING TUMOR SUPPRESSOR ACTIVITY OF PTSG.

The tumor suppressor activity of PTSG is assessed in both in vitro cell culture conditions and in nude mouse animal models. Any of the 13 N33-colon carcinoma cell lines listed in Figure 17 (SW480, SW837, SW1417, HT-29, SW403, LS174T, DLD-1, CACO-2, EB, SK-CO-1, RKO, HCT116 and COLO-302) can be used to assess PTSG tumor suppressor activity.

Briefly, the effect of PTSG on the proliferation of the above cell lines is assessed following expression of PTSG using a adenoviral expression vector. ACN is a control adenoviral vector lacking a cDNA insert while AC-PTSG are adenoviral vectors expressing PTSG products under the control of the human CMV promoter.

In Vitro Transcription Translation of PTSG

Plasmid pBS-N33C(7) was tested for the ability to produce a 39 KD protein in the TNT Coupled Reticulocyte Lysate System (Promega, Madison, Wisconsin). The T7 promoter in the Bluescript vector (Stratagene) allows for transcription and translation of the PTSG coding sequence by rabbit reticulocytes. One microgram of mini-lysate DNA is added per TnT Reticulocyte reaction and is incubated for 1 hour at 30 degrees Celsius. Ten microliters of the reaction is mixed with loading buffer and run on a 10% polyacrylamide gel (Novex) for 1 1/2 hour at 165 V. The gel is dried down and exposed to film overnight.

Construction of adenoviral vectors containing PTSG

To construct recombinant adenoviruses, the insert of pBS-N33C(7) was recovered by EcoRI digestion and cloned into the EcoRi site of pcDNA3 (Invitrogen) to yield pcDNA3-N33 clones. The orientation of the insert was tested by Kpn I digestion, and clones in antisense orientation relative to the CMV promoter in pcDNA3 were

subsequently used. For construction of the Form 1 adenovirus, pcDNA3-N33 was digested with Xba I - BamH I, and the insert was directionally cloned into the Xba I - BamH I sites of pAdCMVb vector to yield pACN33-1. For construction of the Form 2 adenovirus, pBS-N33C(7) was digested with ava III - EcoR I and the 5' half (nt 1 - 616) of N33 insert was purified. Clone A5 was also digested with Ava III - EcoR I to release the 3' half of the N33 Form 2 insert (nt 617 - EcoRI). The two gene halves were ligated and cloned into the EcoR I site of pcDNA3. Orientation of the reconstructed insert was agsin tested by Kpn I digestion and sequencing. An antisense orientation clone was then cut with Xba I and Bam HI and the insert cloned into pAdCMVb as above to yield pACN33-2.

The above plasmids are linearized with Nru I and are co-transfected with the large fragment of a Cla I digested d1309 mutants (Jones and Shenk, Cell, 17:683-689 (1979) which is incorporated herein by reference), using a $CaPO_4$ transfection kit (Stratagene). Viral plaques are isolated and recombinants are identified by both restriction digest analysis and PCR using primers against PTSG cDNA sequence. Recombinant virus is further purified by limiting dilution, and virus particles were purified and titered by standard methods (Graham and van der Erb, Virology, 52:456-457 (1973); Graham and Prevec, Manipulation of Adenovirus Vectors. In: Methods in Molecular Biology Vol 7: Gene Transfer and Expression Protocols, Murray E.J. (ed.) The Humana Press Inc.,

Clifton N.J., 7:109-128 (1991), both of which are incorporated herein by reference).

To ensure that the PTSG vector above expresses a protein of the appropriate size, colon carcinoma cell lines are infected with either the control or the PTSG-containing recombinant adenoviruses for a period of 24 hours at increasing multiplicities of infection (MOI) of plaque forming units of virus/cell. Cells are then washed once with PBS and harvested in lysis buffer (50mM Tris-Hcl Ph 7.5, 250 Mm NaCl, 0.1% NP40, 50mM NaF, 5mM EDTA, 10ug/ml aprotinin, 10 ug/ml leupeptin, and 1mM PMSF). Cellular proteins are separated by 10% SDS-PAGE and transferred to nitrocellulose. Membranes are incubated with an anti-PTSG antibody followed by sheep anti-mouse IgG conjugated with horseradish peroxidase. Accurate expression of PTSG protein is visualized by chemiluminescence (ECL kit, Amersham) on Kodak XAR-5 film.

In Vitro.

N33-negative colon cancer cells (selected from the cell lines set out in Figure 17) are seeded at $1x10^6$ cells per 100 mm plate in Kaighn's F12/DME medium (Irvine Scientific) which is supplemented with 10% FBS and 0.2 IU insulin (Sigma). The plates are incubated overnight at 37°C in 7% CO_2. The following day, the cells are refed with 10 mls of growth medium and are infected with either ACN control viral lysate (MOI 10) or with AC-PTSG viral lysates (MOI 10) and allowed to incubate at 37°C. After

3 days, the medium is removed and the cells are fixed with a 1:5 acetic acid-methanol solution. The cells are stained with a 20% methanol-0.5% crystal violet solution for 30 minutes and are rinsed with tap water to remove excess stain.

Thymidine incorporation is also used to assess the effects of PTSG on cell proliferation. Briefly, approximately $3x10^3$ cells are plated in each well of a 96-well plate (Costar) and allowed to incubate overnight (37°C, 7% CO_2). Serial dilutions of ACN or AC-PTSG are made in DME:F12/15% FBS/1% glutamine, and cells are infected at multiplicity of infection (MOI) of 10 and 100 (4 replicate wells at each MOI) with each adenovirus. One-half of the cell medium volume is changed 24 hours after infection and every 48 hours until harvest. At 18 hours prior to harvest, 1 μCi of ^{3}H-thymidine (Amersham) is added to each well. Cells are harvested onto glass-fiber filters 5 days after infection, and ^{3}H-thymidine incorporated into cellular nucleic acid is detected using liquid scintillation (TopCount®, Packard Instruments). Cell proliferation (cpm/well) at each MOI is expressed as a percentage of the average proliferation of untreated control cells.

Ex Vivo Gene Therapy.

To assess the effect of PTSG expression on tumorigenicity, the above tumor cell lines are tested for their ability to produce tumors in nude mouse models. Approximately $2x10^7$ cells are plated into T225 flasks, and

cells are treated with sucrose buffer containing ACN or AC-PTSG adenoviruses at MOI of 3 or 30. Following overnight infections, cells are harvested and approximately 10^7 cells are injected subcutaneously into the left and right flanks of BALB/c nude mice (4/group) that had previously received subcutaneous pellets of 17ß-estradiol. One flank is injected with ACN-treated cells, while the contralateral flank is injected with AC-PTSG treated cells, each mouse serving as its own control. Animals receiving bilateral injections of untreated cells serve as an additional control for tumor growth. Tumor dimensions (length, width, height) and body weights are then measured twice per week. Tumor volumes are estimated for each animal assuming a spherical geometry with radius equal to one-half the average of the measured tumor dimensions.

In Vivo Tumor Suppression with PTSG.

Colon cancer cell lines are injected subcutaneously into female BALB/c athymic nude mice. Tumors are allowed to develop for 32 days. At this point, a single injection of either ACN (control) or AC-PTSG adenoviruses are injected into the peritumoral space surrounding the tumor. Tumors are then excised at either Day 2 or Day 7 following the adenovirus injection, and poly-A+ RNA is isolated from each tumor. Reverse transcriptase-PCR using PTSG specific primers, are then used to detect PTSG RNA in the treated tumors. Amplification with actin primers will serve as a control for the RT-PCR reaction while a plasmid containing the

recombinant-(PTSG) sequence will serve as a positive control of the recombinant-(PTSG) specific band.

In a separate experiment, cells are injected into the subcutaneous space on the right flank of mice, and tumors are allowed to grow for 2 weeks. Mice receive peritumoral injections of buffer or recombinant virus twice weekly for a total of 8 doses. Tumor growth is monitored throughout treatment in the control animals receiving ACN and buffer and those animals receiving AC-PTSGs. Body weight and survival time is also monitored.

sAlthough the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

Table 1
CHROMOSONE 8p DELETION MAP IN HUMAN PROSTRATE CANCER
Allelic Loss on Chromosone 8 in Prostrate Cancer

Locus	Polymorphism type	Probe	Enzyme	Location	No. of Cases	Allelic losses/ informative cases (all tumors)	Allelic losses/ informative cases node metastases)
D8S140	RFLP[a]	C18-1	MspI	8p23.2-23.3	49	4/28 (11)[b]	1/6 (17)
D8S201	Micro-satellite	Mfc199	--	8p23	30	3/22 (14)	1/2 (50)
D8S163	RFLP	KSR2	TaqI	8p22-pier	50	14/23 (61)	2/4 (50)
MSR	RFLP	M8R32	MspI	8p22	50	20/29 (69)	3/3 (100)
LPL	Micro-satellite	GZ14.15	--	8p22	45	15/32 (47)	0/2 (0)
D8S220	RFLP	C18-319	TaqI	8p21.2-21.3	51	16/27 (59)	4/5 (80)
NEFL	RFLP	NF5.1	TaqI	8p21	12	2/6 (33)	Not studied
D8S194	RFLP	C18-277	MspI	8p11.21-11.22	51	3/20 (12)	1/4 (25)
D8S39	RFLP	MCT128.2	TaqI	8q24	43	2/17 (12)	0/3 (0)

[a] RFLP, restriction fragment length polymorphism.

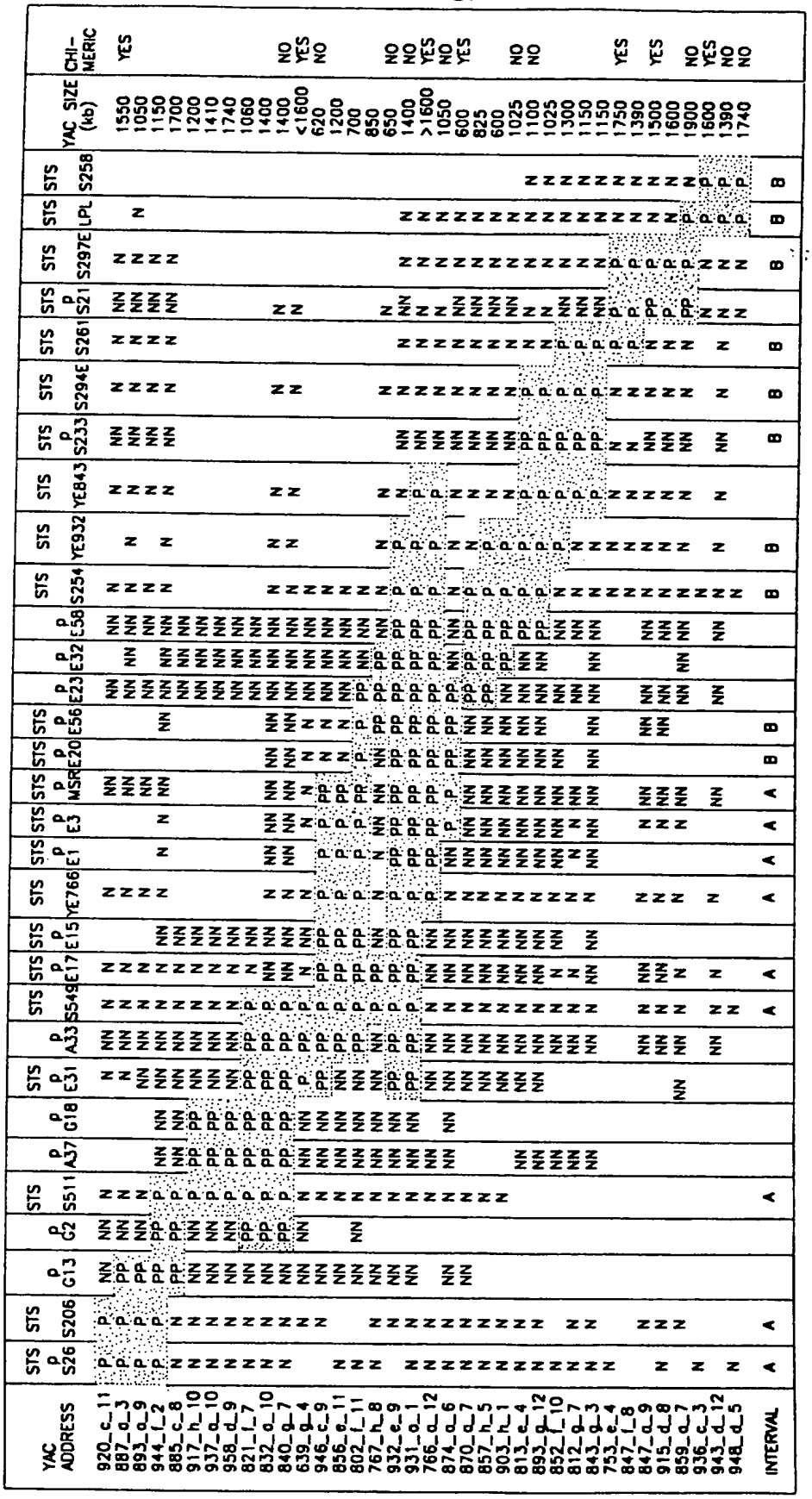
[b] Number in parentheses, percentage

No.	Locus name	Chromosomal interval	Type	Primer sequences (5'-3')	Product size (bp)	Anneal temp. (°C)	Reference
1	D8S26	A	RFLP	TAGCTCCTTCGAAACCCTCA TGGCAGGAAAAGCTCTCAAT	124	60	This report+
2	D8S511	A	STRP	TTGTCCCTGTTGGCAGA TGATTTTTGTGTCCTGAAACTTA	~135	55	This report
3*	D8S549	A	STRP	AAATGAATCTCTGATTAGCCAAC TGAGAGCCAACCTATTTCTACC	~170	55	This report
4	MSR	A	RFLP	TTCATCTATTGCATTCC CAAAATTTCAGCATGACAACTG	102	50	Matsumoto et al., 1990
5	D8S254	B	STRP	TGCCGGACATACATTAGTGA TTGTAAACACCACAAGCAGG	~70	55	J. Weber, pers. commun.
6	D8S233	B	RFLP	TTTGAGTAGCCAGAGTCCAG CGTACCATTTCCATCTGCT	84	55	This report+
7	D8S261	B	STRP	TGCCACTGTCTTGAAAATCC TATGGCCCAGCAATGTGTAT	~135	55	Weissenbach et al., 1992
8	D8S21	B	RFLP	CACTGAGGAAGAGGTTGAAG ATCCATCACCAGGTTTGG	86	55	This report+
9	LPL	B	STRP	ATCTGACCAAGGATAGTGGGAT CCTGGGTAACTGAGCGAGACT	~130	60	Zuliani and Hobbs, 1990
10*	D8S258	B	STRP	CTGCCAGGAATCAACTGAG TTGACAGGGACCCACG	~150	55	Weissenbach et al., 1992

Table 2. Polymorphic loci on chromosome arm 8p comprising mapping framework. Chromosomal intervals (A or B) are defined as in Wagner et al. (1991). All loci were used to screen YAC pools. *: Screening performed at Genethon. +: STS created within RFLP probe as described herein.

No.	Locus/probe Name	Chromosomal Interval	Type	Primer Sequences (5'-3')	Product Size	Anneal temp (°C)
1	D8S206	A	STRP	GAAAACCATGGCTGGGTG ACATGCATTAGCACTACCATGC	~130	55
2	D8S294E	B	EST	TGACCTGAAATTACAAGGTA AGCAGCTTGACAATCTTAAG	82	55
3	D8S297E	B	EST	CGTAGCTGCAGTTGTCCACG CATTCTGACTACTACTTTCAG	67	55
4	E1*	A	random subclone	TGACACACTTGCCATTTGAT TTCCATTAGTCCCAGTTGTC	131	55
5	E3	A	random subclone	GCCTGTTTCATCGAACC CCTGGCATTCTTTACCTAGA	85	55
6	E15	A	random subclone	GTTCTTGCCATGTGATGTG GTGGCATCTGCTTCTGG	86	55
7	E17	A	random subclone	CAAGGCATATCACAACTGC GATAATTGAACTGTCACCTCTG	121	55
8	E20	B	random subclone	TGAATTTGCATAGTCTGCAG CAGCTCTAACAAGGCTCCTA	107	55
9	E31	A	random subclone	TCAGGGCCTCTTGCAT TGGGAACTTCAAGCATAGG	97	55
10	E56	B	random subclone	TTTGTTGAGGACAAATACCC TGTCACGATGAGGATTGTTA	170	55
11	YE766	A	YAC end	GACTCTTGCCACCTTGTAAA ATCTCCAAACCTACTTCTCC	89	55
12	YE843	B	YAC end	AGCAAAGTGATGGTGGTAAC GGACTAATTACCTCAGGCCT	82	55
13	YE932	B	YAC end	ATGGAAATGCACGGGA CCATTCTGTCCCAATGATC	173	55

Table 3. Sequence-tagged sites used for physical map refinement. Chromosomal intervals (A or B) are defined as in Wagner et al. (1991). D8S206 (Hudson et al., 1992), D8S294E and D8S297E (Adams et al., 1992) were reported previously; remaining STSs were created by partial sequencing of subcloned probes as described herein. *Detects Hind III RFLP. EST: expressed sequence tag.



YAC ADDRESS	STS p S26	STS S206	p G13	p G2	STS S511	p A37	p G18	STS p E31	p A33	STS S549	STS p E17	STS p E15	STS YE766	STS p E1	STS p E3	STS p MSRE20	STS p E56	p E23	p E32	p E58	STS S254	STS YE932	STS YE843	STS p S233	STS S294E	STS S261	STS p S21	STS S297E	STS LPL	STS S258	YAC SIZE (kb)	CHI-MERIC	
920_c_11	P	P	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N			NN	NN	NN	N	N	N	NN	N	N	N	N	N		1550	YES	
887_a_3	P	P	PP	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N			NN	NN	NN	N	N	N	NN	N	N	N	N	N		1050	YES	
893_a_9	P	P	PP	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N			NN	NN	NN	N	N	N	NN	N	N	N	N	N		1150	YES	
944_f_2	P	P	PP	PP	P	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	NN	N	N	N	N	N		1700	YES	
885_c_8	N	N	NN	PP	P	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	NN	N	N	N	N	N		1200	YES	
917_h_10	N	N	NN	NN	P	PP	PP	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	NN	N	N	N	N	N		1410	YES	
937_a_10	N	N	NN	NN	P	PP	PP	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	NN	N	N	N	N	N		1740	YES	
958_d_9	N	N	NN	NN	P	PP	PP	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	NN	N	N	N	N	N		1060	YES	
821_f_7	N	N	NN	PP	P	PP	PP	N	PP	P	PP	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1400	NO	
832_a_10	N	N	NN	PP	P	PP	PP	N	PP	P	PP	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1400	YES	
840_g_7	N	N	NN	PP	P	PP	PP	N	PP	P	PP	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		<1600	NO	
639_g_4	N	N	NN	NN	N	NN	NN	N	PP	P	PP	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		620		
946_c_9	N	N	NN	NN	N	NN	NN	N	PP	P	PP	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1200	NO	
856_e_11	N	N	NN	NN	N	NN	NN	N	PP	P	PP	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		700	YES	
802_f_11	N	N	NN	NN	N	NN	NN	N	PP	P	PP	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		850	NO	
767_h_8	N	N	NN	NN	N	NN	NN	N	PP	P	PP	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		650	NO	
932_e_9	N	N	NN	NN	N	NN	NN	N	PP	P	PP	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1400	YES	
931_a_1	N	N	NN	NN	N	NN	NN	N	PP	P	PP	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		>1600	NO	
766_a_12	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	NN	N	N	N	N	N		1050	YES	
874_a_6	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	NN	N	N	N	N	N		600	NO	
870_a_7	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	NN	N	N	N	N	N		825	YES	
857_h_5	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	NN	N	N	N	N	N		600	NO	
903_h_1	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	NN	N	N	N	N	N		1025	NO	
813_e_4	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1100	NO	
893_g_12	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1025	NO	
852_f_10	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1300	YES	
812_g_7	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1150	YES	
843_g_3	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1150	YES	
753_e_4	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1750	YES	
847_f_8	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1390	YES	
847_a_9	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1500	YES	
915_d_8	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1600	NO	
859_a_7	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1900	YES	
936_c_3	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1600	NO	
943_d_12	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1390	NO	
948_d_5	N	N	NN	NN	N	NN	NN	N	NN	N	NN	NN	N	NN	N		NN	NN	NN	NN	N	N	N	PP	N	N	N	N	N		1740	NO	
INTERVAL	A	A			A					A	A		A	A	A	B	B				B	B	B	B	B	B	B	B	B	B			

TABLE 4

Marker A	Marker B	Number of clones observed ++	+-	-+	--	total	Theta	cR_{5000}	LOD
D8S26	MSR	11	5	3	73	92	0.3115	37	7.17
D8S26	D8S233	9	6	2	74	91	0.3394	41	5.99
D8S26	D8S261	9	7	1	75	92	0.3463	43	6.08
D8S26	D8S21	10	6	1	75	92	0.2948	35	7.21
D8S26	LPL	10	6	3	73	92	0.3595	45	6.08
MSR	D8S233	11	3	1	77	92	0.1741	19	9.87
MSR	D8S261	9	6	2	76	93	0.3563	44	6.06
MSR	D8S21	10	5	2	76	93	0.3026	36	7.20
MSR	LPL	10	5	4	74	93	0.3675	46	6.10
D8S233	D8S261	9	3	1	79	92	0.1995	22	8.31
D8S233	D8S21	10	2	1	79	92	0.1444	16	9.89
D8S233	LPL	9	3	4	76	92	0.3147	38	6.45
D8S261	D8S21	11	0	1	81	93	0.0496	5	12.91
D8S261	LPL	10	1	4	78	93	0.2305	26	8.46
D8S21	LPL	11	1	3	78	93	0.1786	20	9.89

Table 5. Pairwise analysis of six markers in the radiation hybrid panel. Statistics were calculated by the TWOPOINT program. cR_{5000}: centiray at 5000 Rad irradiation.

Genomic subclones

series	Name	Probe fragments, kb (enzyme)	Human chrom. 8	HindIII fragments detected, kb
932E9				
E	1c	1.6 (EcoRI)	Yes	8
E	1e	1.0 (EcoRI)	Yes	[4 + 8], 12**
E	2d	0.85, 1.0 (EcoRI)	Yes	7.5
E	2	0.8 (EcoRI)	Yes	5.5 + 3.8
E	3	0.5, 4, 5 (EcoRI+NotI)	Yes	~12
E	6*	1.2 (EcoRI+NotI)	Yes	3.8
E	10*	0.25, 3.0 (EcoRI+NotI)	Yes	7
E	15	0.9 (HindIII+SacII)	Yes	1.2
E	17*	0.3, 1.2 (EcoRI+NotI)	Yes	5
E	18	1.0, 1.8, 4.5 (HindIII)	Yes	1.0
E	20*	1.5, 1.9 (EcoRI+Not)	Yes	7
E	23*	1.6 (EcoRI+NotI)	Yes	8
E	31*	1.9, 2.5, 3 (EcoRI+NotI)	Yes	3.5
E	32	0.2, 0.5, 1.0, 2.1 (EcoRI)	Yes	~8
E	56	0.2, 0.4, 1 (EcoRI+StyI)	Yes	5.6
E	58	0.8, 1.2, 1.8 (Eco+SacII)	Yes	8.5
767H8				
H	23	1, 1.3, 2.1 (EcoRI+SacII)	Yes	6
H	25	0.5, 1.7 (PstI+SacII)	Yes	1.6
H	29	1.0, 1.5 (EcoRI+SacII)	Yes	4.2, 2.4, 1.3??
H	31	0.45 (EcoRI+SacII)	Yes	7
802F11				
F	4	0.5, 1.6 (EcoRI+SacII)	Yes	1.6
832A10				
A	33	0.35 (EcoRI+SacI)	Yes	1
A	37	1.1, 3.2 (EcoRI+SacII)	Yes	12
821F7 & 885C8				
G	2	0.5, 6 (HindIII+SacII)	Yes	~1.2
G	4	0.4 (SmaI)	Yes	3.2 + high background
G	10	0.25, 6 (EcoRI+SacII)	Yes	~12
G	14	0.4, 1.6 (EcoRI+SacII)	Yes	~2
G	18*	1.2 (EcoRI+SacII)	Yes	~10
YAC end clones				
YE1-766A12		0.25 (EcoRI)	Yes	5
YE1-843G3			Yes	
YE1-932E9		0.6 (EcoRI)	Yes	1.8
PAC E1c subclones				
PAC A2			Yes	
PAC A3			Yes	
PAC B3			Yes	

*Contains no Alu; **polymorphism; fragment without Alu used as probe.

TABLE 6

TABLE 7

selected cDNAs

Series	No.	Probe fragments, bp (enzyme)	Human chrom. 8	HindIII frag's detected, kb	ID or Hom.
selected by 802F11					
J	2	~325 (BstXI)	Yes	2.5	
J	10	~350 (BstXI)	Yes	4	
J	12	~350 (BstXI)	Yes**	~10* + 6**	hom. to PP2Cα
J	28	~400 (BstXI)	Yes	4 (+ 3, weak)	novel ORF
P	3	~350 (BstXI)	Yes	~12	MSR (1-280 bp)
P	10	~400 (BstXI)	Yes	2	
P	14	~450 (BstXI)	Yes	3.5	
P	16	1 band (BstXI)	Yes	2.2	
P	25	~300 (BstXI)	Yes	4	
P	27	~450+350 (BstXI)	Yes	4	seq. overlap with J28
P	28	~400 (BstXI)	Yes	12 (+ 5.2)	MSR (1-450 bp)
P	34	~250 (BstXI)	Yes	3.8	MSR (3' UTR) & HSDHEHC01
W	17	~400(R1+HIII)	Yes	3.5+background	
selected by 821F7 or 877F2					
K	26	~350 (RI+HIII)	Yes	>12	
K	27	~500 (RI+HIII)	Yes	>12	
K	36	~250 (RI+HIII)	Yes	2.7	
selected by 946C9					
L	3	~400 (RI+HIII)	Yes	4	overlaps J28
L	5	~325 (RI+HIII)	Yes	0.5	
L	12	~300 (RI+HIII)	Yes	3.8	
L	14	~550 (RI+HIII)	Yes	6 + some background	
L	21	~450 (RI+HIII)	Yes	5.4+4+3	
L	30	(RI+HIII)	Yes	4.5 + some background	
L	31	(RI+HIII)	Yes	7	
N	1	(RI+HIII)	Yes	4.8 + high background	
N	7	(RI+HIII)	Yes	3 + background	
N	14	~600 (RI+HIII)	Yes	2.6	
N	18	~250 (RI+HIII)	Yes	>12	
N	19	~600 (RI+HIII)	Yes	1.6	
N	21	~800 (RI+HIII)	Yes		overlaps L21
N	27	~500 (RI+HIII)	Yes	3+ background	
N	28	~550 (RI+HIII)	Yes	4	overlaps J28
N	33	(RI+HIII)	Yes	12,11,4,3.2,2	overlaps L21
N	35	(RI+HIII)	Yes	~12	
N	36	(RI+HIII)	Yes	12,11,4,3,2	overlaps N33
X	3	~500 (RI+HIII)	Yes	~12	
X	6	~500 (RI+HIII)	Yes	1.8	
selected by 932E9					
Q	30	~500 (RI+HIII)	Yes	3.5	
selected by 946C9 and 932E9					
Y	1A1	U & L bands	Yes		
Y	1A8		Yes		
Y	1C8		Yes		

* polymorphic; ** 6 kb band is not on chromosome 8.

What is claimed is:

1. An isolated and purified DNA sequence encoding a prostate tumor suppressor protein comprising a nucleotide sequence of SEQ ID NO: 1.

2. An isolated and purified DNA sequence encoding a prostate tumor suppressor protein comprising a nucleotide sequence of SEQ ID NO: 2.

3. A recombinant vector containing the isolated, purified DNA of claims 1 or 2.

4. A recombinant vector of claim 3, wherein the vector is a cosmid, plasmid, or is derived from a virus.

5. An expression vector comprising said DNA molecule of claims 1 or 2, capable of inserting said DNA molecule into a mammalian host cell and of expressing the protein therein.

6. An expression vector of claim 5, wherein said expression vector is selected from the group consisting of a plasmid and a viral vector.

7. An expression vector of claim 6, wherein the vector is a viral vector and is selected from the group consisting of a retroviral vector and an adenoviral vector.

8. An expression vector of claim 7, wherein said expression vector is an adenoviral vector.

9. A host-vector system for the production of a polypeptide or protein having the biological activity of an PTSG protein or biologically active derivative thereof which comprises the vector of claims 5, 6, 7 or 8 in a suitable host cell.

10. A host-vector system of claim 9, wherein the host cell is a prokaryotic cell.

11. A host-vector system of claim 10, wherein the host cell is a eukaryotic cell.

12. A pharmaceutical composition comprising the vector of claim 5 and a pharmaceutically-acceptable carrier.

13. A pharmaceutical composition comprising the vector of claim 6 and a pharmaceutically-acceptable carrier.

14. A pharmaceutical composition comprising an AC-PTSG vector and a pharmaceutically acceptable carrier.

15. A DNA probe comprised of at least about 15 nucleotides complementary to the DNA sequence of claim 1.

16. A DNA probe comprised of at least about 15 nucleotides complementary to the DNA sequence of Claim 2.

17. An isolated and purified mammalian protein comprising an amino acid sequence of SEQ ID NO: 3.

18. An isolated and purified mammalian protein comprising an amino acid sequence of SEQ ID NO: 4.

19. A method of producing a protein of claim 17 or 18 comprising the steps of:

a. inserting a compatible expression vector comprising a gene encoding a protein of claim 17 or claim 18 into a host cell;

b. causing said host cell to express said protein.

20. A method according to claim 19, wherein said host cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.

21. A method according to claim 20, wherein said host cell is a eukaryotic host cell which is a mammalian host cell and said expression vector is compatible with said mammalian host cell.

22. A method of suppressing the neoplastic phenotype of a cancer cell having no endogenous PTSG protein comprising administering to such cancer cell an effective amount of the DNA of claims 1 or 2.

23. The method of claim 22, wherein the administering of the PTSG gene is by recombinant vector.

24. A method of suppressing the neoplastic phenotype of a cancer cell lacking endogenous wild-type PTSG product comprising administering to such cancer cell the protein of claims 17 or 18.

25. An antibody which binds a PTSG peptide which peptide is comprised of a sequence of the protein of SEQ ID NO: 3.

26. An antibody of claim 25, which binds to the PTSG protein having the amino acid sequence of SEQ ID NO: 3.

27. An antibody which binds a PTSG peptide which peptide is comprised of a sequence of the protein of SEQ. ID No. 4.

28. An antibody of claim 27, which binds to the PTSG protein having the amino acid sequence SEQ. ID No. 4.

29. A method of detecting the absence of PTSG protein in tumor cells, comprising the steps of;

a. preparing tissue sections from a tumor;

b. contacting the antibody of claims 23 or 24 with said tissue sections; and

c. detecting the presence or absence of said antibody binding to said tissue sections.

30. An RNA probe comprised of at least about 15 nucleotides complementary to the DNA sequence of claim 1.

31. An RNA probe comprised of at least about 15 nucleotides complementary to the DNA sequence of claim 2.

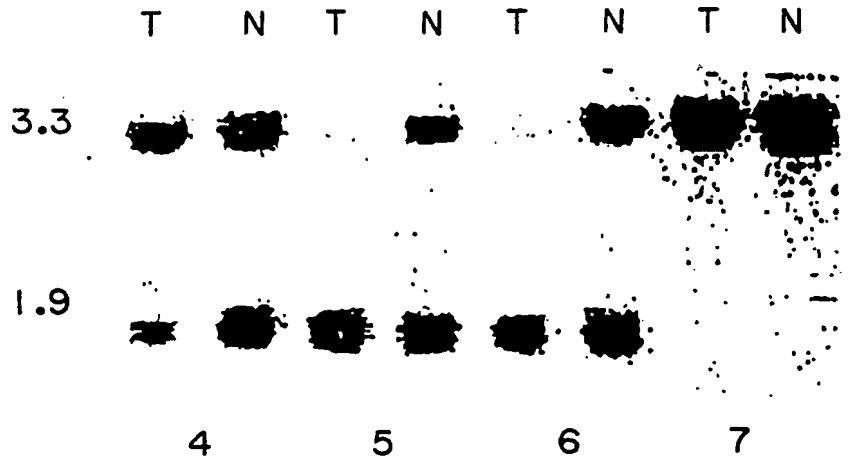

T N T N T N T N
3.3
1.9
4 5 6 7

FIG. 1

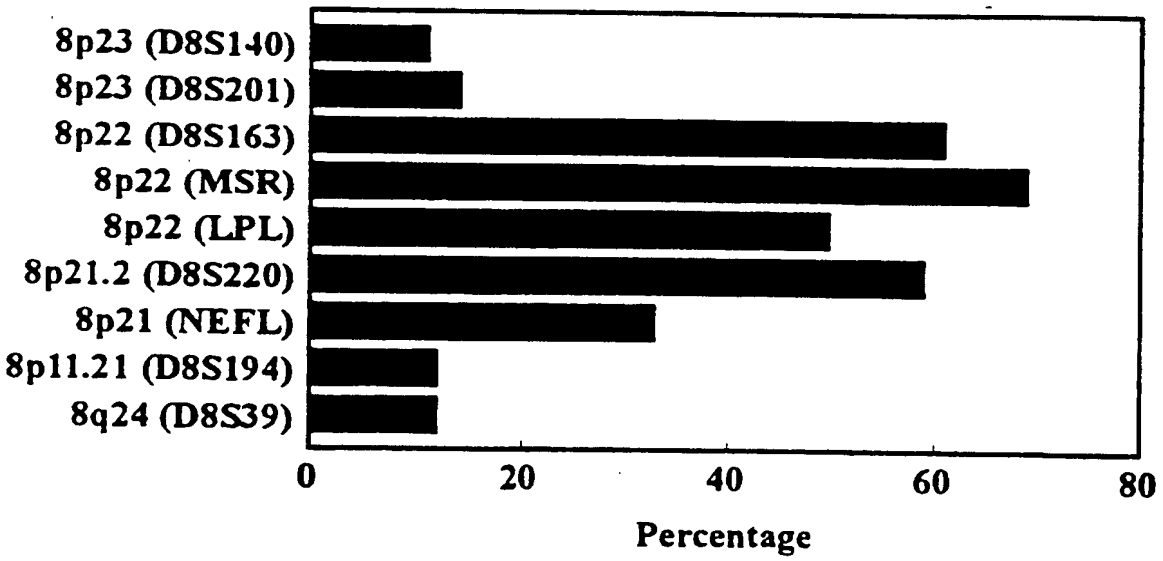

8p23 (D8S140)
8p23 (D8S201)
8p22 (D8S163)
8p22 (MSR)
8p22 (LPL)
8p21.2 (D8S220)
8p21 (NEFL)
8p11.21 (D8S194)
8q24 (D8S39)
0 20 40 60 80
Percentage

FIG. 2

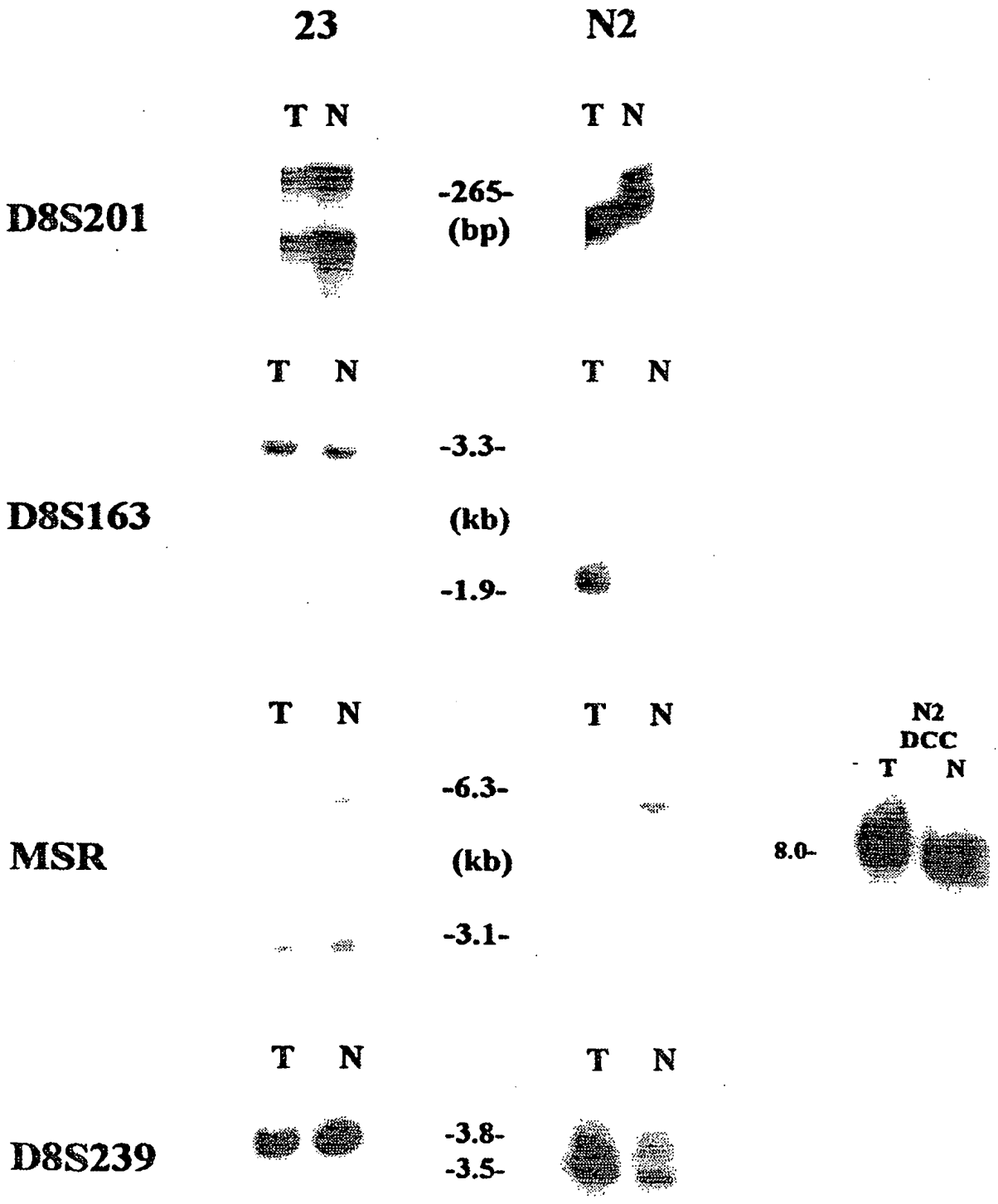

23
N2
T N
T N
D8S201
-265-
(bp)
T N
T N
-3.3-
D8S163
(kb)
-1.9-
T N
T N
N2
DCC
T N
-6.3-
MSR
(kb)
8.0-
-3.1-
T N
T N
D8S239
-3.8-
-3.5-

FIG. 3

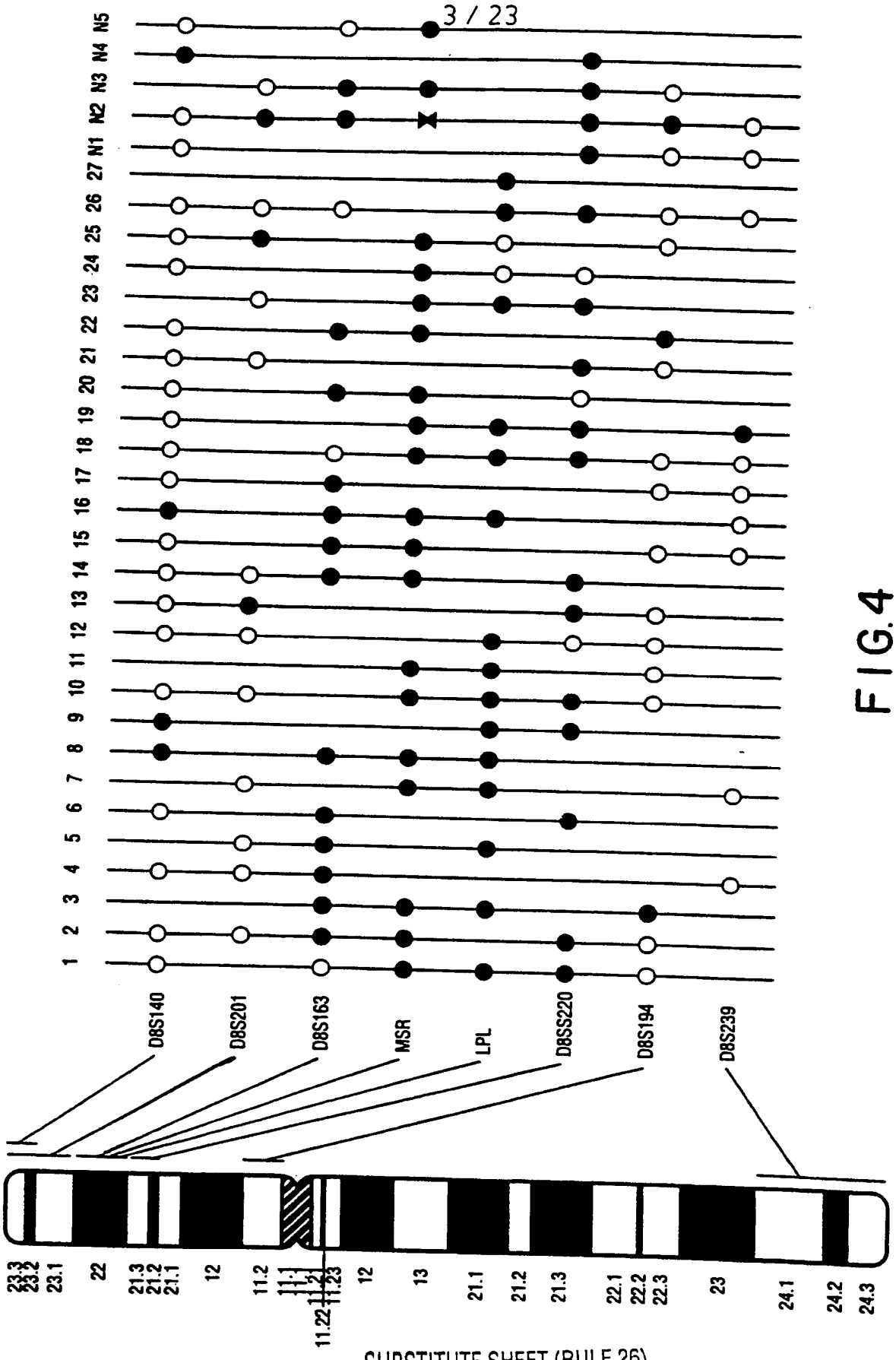
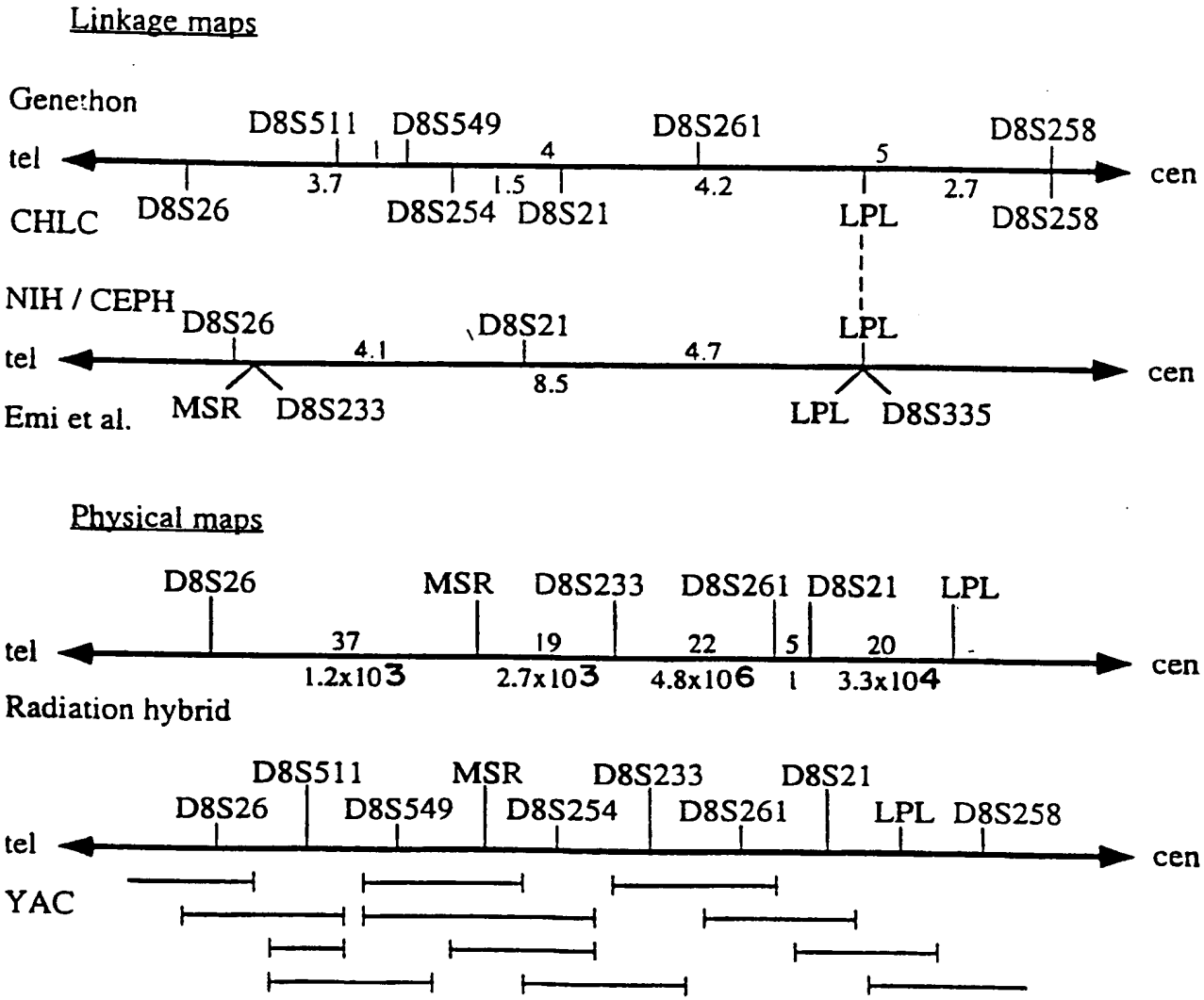

N5
N4
N3
N2
N1
27
26
25
24
23
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
D8S140
D8S201
D8S163
MSR
LPL
D8SS220
D8S194
D8S239
23.3
23.2
23.1
22
21.3
21.2
21.1
12
11.2
11.1
11.21
11.22
11.23
12
13
21.1
21.2
21.3
22.1
22.2
22.3
23
24.1
24.2
24.3

FIG.4


Linkage maps
Genethon
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D8S511
D8S549
D8S261
D8S258
3.7
1.5
4
4.2
5
2.7
cen
CHLC
D8S26
D8S254
D8S21
LPL
D8S258
NIH / CEPH
D8S26
D8S21
LPL
tel
4.1
4.7
8.5
cen
Emi et al.
MSR
D8S233
LPL
D8S335
Physical maps
D8S26
MSR
D8S233
D8S261
D8S21
LPL
tel
37
19
22
5
20
cen
1.2x103
2.7x103
4.8x106
1
3.3x104
Radiation hybrid
D8S511
MSR
D8S233
D8S21
D8S26
D8S549
D8S254
D8S261
LPL
D8S258
tel
cen
YAC

FIG.5

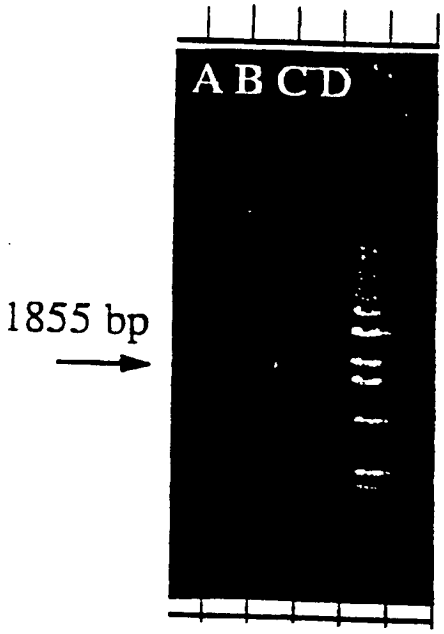
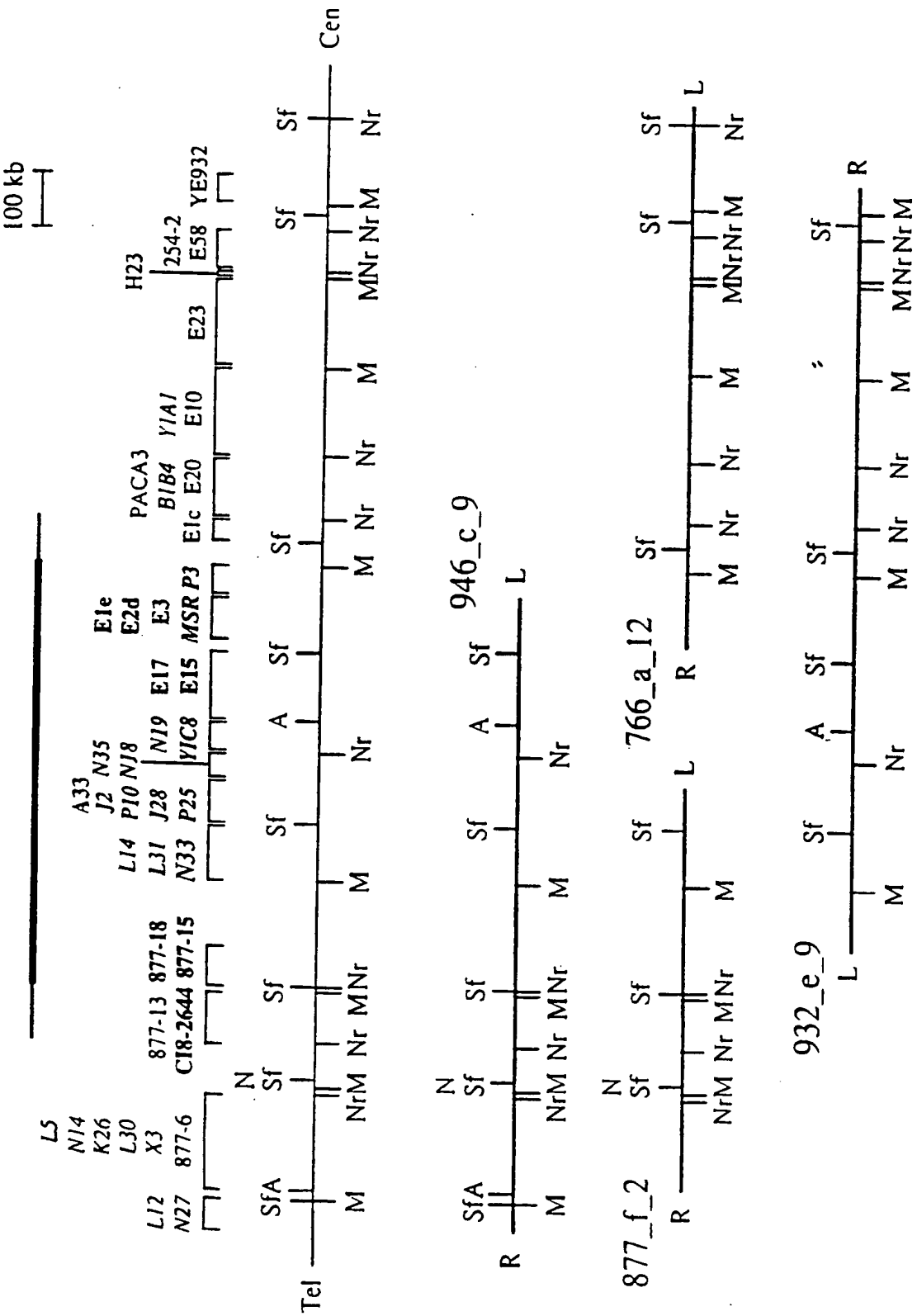


FIG.6

FIG.7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Cen
L5
N14
K26
L30
X3
877-6
L12
N27
877-13
CI8-2644
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L14
L31
N33
A33
J2
P10
J28
P25
N35
N18
N19
YIC8
E17
E15
E1e
E2d
E3
MSR P3
PACA3
B184
E1c
E20
Y1A1
E10
E23
H23
254-2
E58
YE932
N
SfA
M
NrM
Sf
Nr
MNr
A
Nr
M
946_c_9
R
L
766_a_12
877_f_2
932_e_9

FIG.8

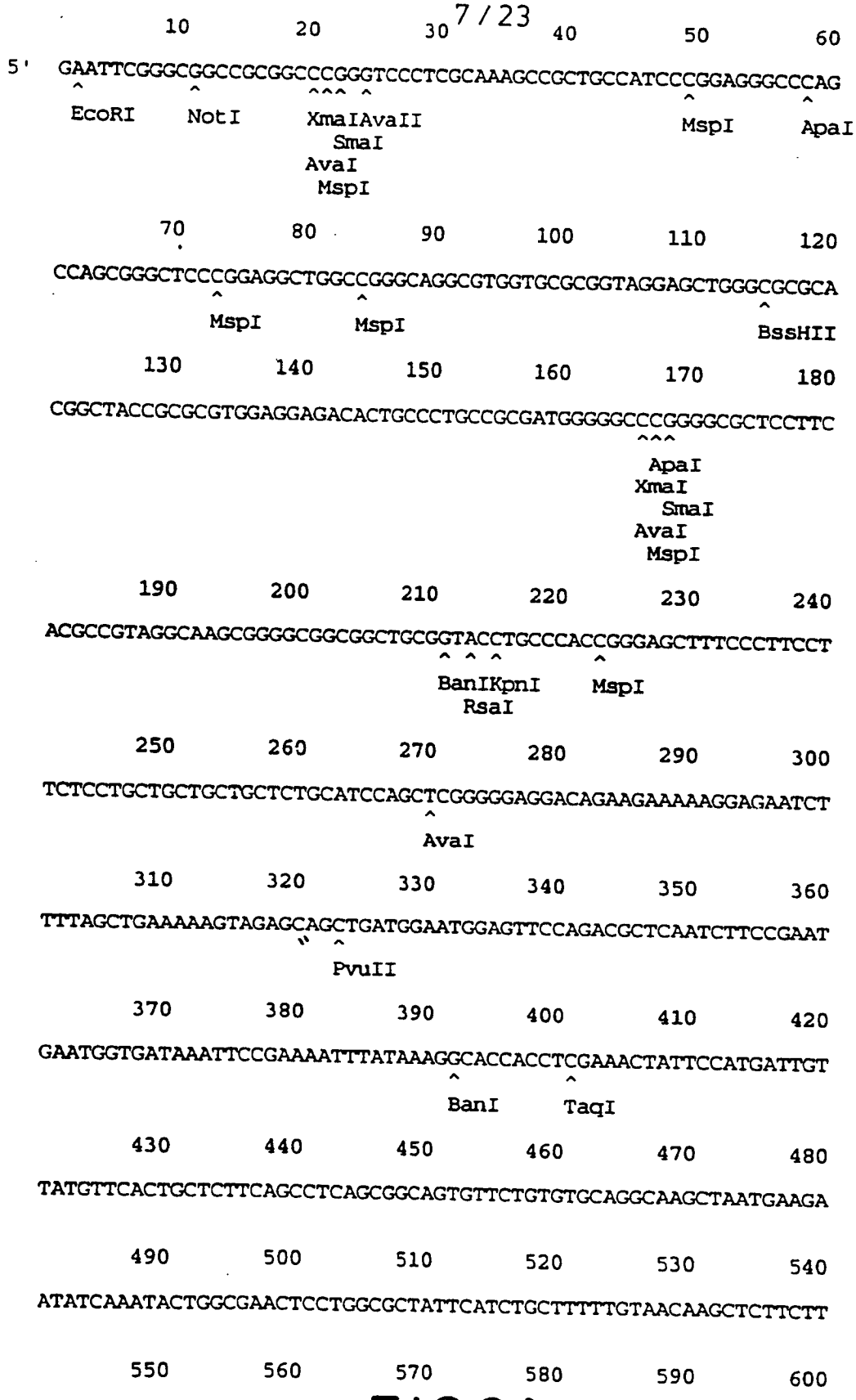

10 20 30 40 50 60
5' GAATTCGGGCGGCCGCGGCCCGGGTCCCTCGCAAAGCCGCTGCCATCCCGGAGGGCCCAG
EcoRI NotI XmaI AvaII SmaI AvaI MspI MspI ApaI
70 80 90 100 110 120
CCAGCGGGCTCCCGGAGGCTGGCCGGGCAGGCGTGGTGCGCGGTAGGAGCTGGGCGCGCA
MspI MspI BssHII
130 140 150 160 170 180
CGGCTACCGCGCGTGGAGGAGACACTGCCCTGCCGCGATGGGGGCCCGGGGCGCTCCTTC
ApaI XmaI SmaI AvaI MspI
190 200 210 220 230 240
ACGCCGTAGGCAAGCGGGGCGGCGGCTGCGGTACCTGCCCACCGGGAGCTTTCCCTTCCT
BanI KpnI RsaI MspI
250 260 270 280 290 300
TCTCCTGCTGCTGCTGCTCTGCATCCAGCTCGGGGGAGGACAGAAGAAAAAGGAGAATCT
AvaI
310 320 330 340 350 360
TTTAGCTGAAAAAGTAGAGCAGCTGATGGAATGGAGTTCCAGACGCTCAATCTTCCGAAT
PvuII
370 380 390 400 410 420
GAATGGTGATAAATTCCGAAAATTTATAAAGGCACCACCTCGAAACTATTCCATGATTGT
BanI TaqI
430 440 450 460 470 480
TATGTTCACTGCTCTTCAGCCTCAGCGGCAGTGTTCTGTGTGCAGGCAAGCTAATGAAGA
490 500 510 520 530 540
ATATCAAATACTGGCGAACTCCTGGCGCTATTCATCTGCTTTTTGTAACAAGCTCTTCTT
550 560 570 580 590 600

FIG.9A

```
CAGTATGGTGGACTATGATGAGGGGACAGACGTTTTTCAGCAGCTCAACATGAACTCTGC

      610        620        630        640        650        660
TCCTACATTCATGCATTTTCCTCCAAAAGGCAGACCTAAGAGAGCTGATACTTTTGACCT
                 ^
               AvaIII
      670        680        690        700        710        720
CCAAAGAATTGGATTTGCAGCTGAGCAACTAGCAAAGTGGATTGCTGACAGAACGGATGT
                   ^
                 PvuII
      730        740        750        760        770        780
TCATATTCGGGTTTTCAGACCACCCAACTACTCTGGTACCATTGCTTTGGCCCTGTTAGT
                                      ^  ^  ^
                                   BanIKpnI
                                     RsaI
      790        800        810        820        830        840
GTCGCTTGTTGGAGGTTTGCTTTATTTGAGAAGGAACAACTTGGAGTTCATCTATAACAA

      850        860        870        880        890        900
GACTGGTTGGGCCATGGTGTCTCTGTGTATAGTCTTTGCTATGACTTCTGGCCAGATGTG
               ^
             StyI
      910        920        930        940        950        960
GAACCATATCCGTGGACCTCCATATGCTCATAAGAACCCACACAATGGACAAGTGAGCTA
                 ^
               AvaII
      970        980        990       1000       1010       1020
CATTCATGGGAGCAGCCAGGCTCAGTTTGTGGCAGAATCACACATTATTCTGGTACTGAA
                                                      ^
                                                    RsaI
     1030       1040       1050       1060       1070       1080
TGCCGCTATCACCATGGGGATGGTTCTTCTAAATGAAGCAGCAACTTCGAAAGGCGATGT
               ^                                      ^
             StyI                                   TaqI
     1090       1100       1110       1120       1130       1140
TGGAAAAAGACGGATAATTTGCCTAGTGGGATTGGGCCTGGTGGTCTTCTTCTTCAGTTT

     1150       1160       1170       1180       1190       1200
TCTACTTTCAATATTTCGTTCCAAGTACCACGGCTATCCTTATAGTGATCTGGACTTTGA
                          ^
                        RsaI
     1210       1220       1230       1240       1250       1260
```

FIG.9B

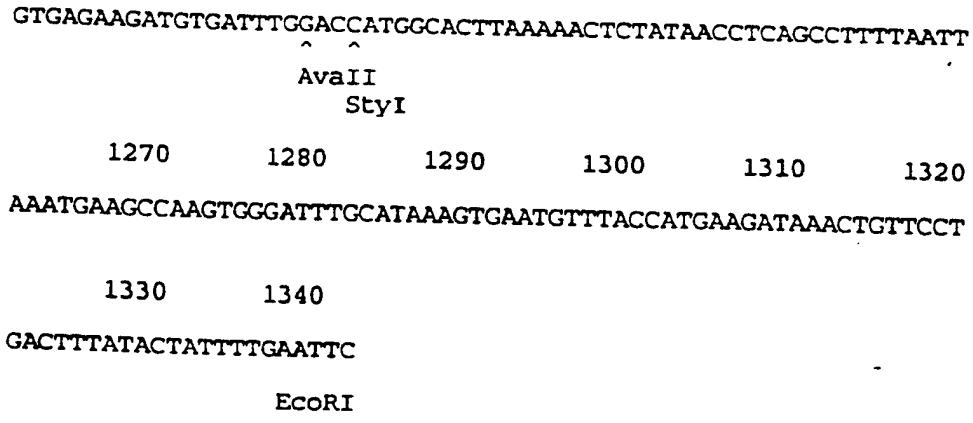

GTGAGAAGATGTGATTTGGACCATGGCACTTAAAAACTCTATAACCTCAGCCTTTTAATT
AvaII
StyI
1270
1280
1290
1300
1310
1320
AAATGAAGCCAAGTGGGATTTGCATAAAGTGAATGTTTACCATGAAGATAAACTGTTCCT
1330
1340
GACTTTATACTATTTTGAATTC
EcoRI

FIG.9C

N33GCR−f

```
        10          20          30          40          50          60
GAATTCGGGC GGCCGCGGCC CGGGTCCCTC GCAAAGCCGC TGCCATCCCG GAGGGCCCAG
CTTAAGCCCG CCGGCGCCGG GCCCAGGGAG CGTTTCGGCG ACGGTAGGGC CTCCCGGGTC

        70          80          90         100         110         120
CCAGCGGGCT CCCGGAGGCT GGCCGGGCAG GCGTGGTGCG CGGTAGGAGC TGGGCGCGCA
GGTCGCCCGA GGGCCTCCGA CCGGCCCGTC CGCACCACGC GCCATCCTCG ACCCGCGCGT

       130         140         150         160         170         180
CGGCTACCGC GCGTGGAGGA GACACTGCCC TGCCGCGATG GGGGCCCGGG GCGCTCCTTC
GCCGATGGCG CGCACCTCCT CTGTGACGGG ACGGCGCTAC CCCCGGGCCC CGCGAGGAAG
                                           START
       190         200         210         220         230         240
ACGCCGTAGG CAAGCGGGGC GGCGGCTGCG GTACCTGCCC ACCGGGAGCT TTCCCTTCCT
TGCGGCATCC GTTCGCCCCG CCGCCGACGC CATGGACGGG TGGCCCTCGA AAGGGAAGGA

       250         260         270         280         290         300
TCTCCTGCTG CTGCTGCTCT GCATCCAGCT CGGGGGAGGA CAGAAGAAAA AGGAGAATCT
AGAGGACGAC GACGACGAGA CGTAGGTCGA GCCCCCTCCT GTCTTCTTTT TCCTCTTAGA
          N33GEX−f
       310         320         330         340         350         360
TTTAGCTGAA AAAGTAGAGC AGCTGATGGA ATGGAGTTCC AGACGCTCAA TCTTCCGAAT
AAATCGACTT TTTCATCTCG TCGACTACCT TACCTCAAGG TCTGCGAGTT AGAAGGCTTA

       370         380         390         400         410         420
GAATGGTGAT AAATTCCGAA AATTTATAAA GGCACCACCT CGAAACTATT CCATGATTGT
CTTACCACTA TTTAAGGCTT TTAAATATTT CCGTGGTGGA GCTTTGATAA GGTACTAACA
                                                    └N33GCR−r
       430         440         450         460         470         480
TATGTTCACT GCTCTTCAGC CTCAGCGGCA GTGTTCTGTG TGCAGGCAAG CTAATGAAGA
ATACAAGTGA CGAGAAGTCG GAGTCGCCGT CACAAGACAC ACGTCCGTTC GATTACTTCT

       490         500         510         520         530         540
ATATCAAATA CTGGCGAACT CCTGGCGCTA TTCATCTGCT TTTTGTAACA AGCTCTTCTT
TATAGTTTAT GACCGCTTGA GGACCGCGAT AAGTAGACGA AAAACATTGT TCGAGAAGAA
                                  N33BP5−f
       550         560         570         580         590         600
CAGTATGGTG GACTATGATG AGGGGACAGA CGTTTTTCAG CAGCTCAACA TGAAGTCTGC
GTCATACCAC CTGATACTAC TCCCCTGTCT GCAAAAAGTC GTCGAGTTGT ACTTGAGACG

       610         620         630         640         650         660
TCCTACATTC ATGCATTTTC CTCCAAAAGG CAGACCTAAG AGAGCTGATA CTTTTGACCT
AGGATGTAAG TACGTAAAAG GAGGTTTTCC GTCTGGATTC TCTCGACTAT GAAAACTGGA

       670         680         690         700         710         720
CCAAAGAATT GGATTTGCAG CTGAGCAACT AGCAAAGTGG ATTGCTGACA GAACGGATGT
GGTTTCTTAA CCTAAACGTC GACTCGTTGA TCGTTTCACC TAACGACTGT CTTGCCTACA

       730         740         750         760         770         780
TCATATTCGG GTTTTCAGAC CACCCAACTA CTCTGGTACC ATTGCTTTGG CCCTGTTAGT
AGTATAAGCC CAAAAGTCTG GTGGGTTGAT GAGACCATGG TAACGAAACC GGGACAATCA
N33BP7−f
       790         800         810         820         830         840
GTCGCTTGTT GGAGGTTTGC TTTATTTGAG AAGGAACAAC TTGGAGTTCA TCTATAACAA
CAGCGAACAA CCTCCAAACG AAATAAACTC TTCCTTGTTG AACCTCAAGT AGATATTGTT

       850         860         870         880         890         900
GACTGGTTGG GCCATGGTGT CTCTGTGTAT AGTCTTTGCT ATGACTTCTG GCCAGATGTG
CTGACCAACC CGGTACCACA GAGACACATA TCAGAAACGA TACTGAAGAC CGGTCTACAC
          N33−f
       910         920         930         940         950         960
GAACCATATC CGTGGACCTC CATATGCTCA TAAGAACCCA CACAATGGAC AAGTGAGCTA
CTTGGTATAG GCACCTGGAG GTATACGAGT ATTCTTGGGT GTGTTACCTG TTCACTCGAT
```

FIG.10A

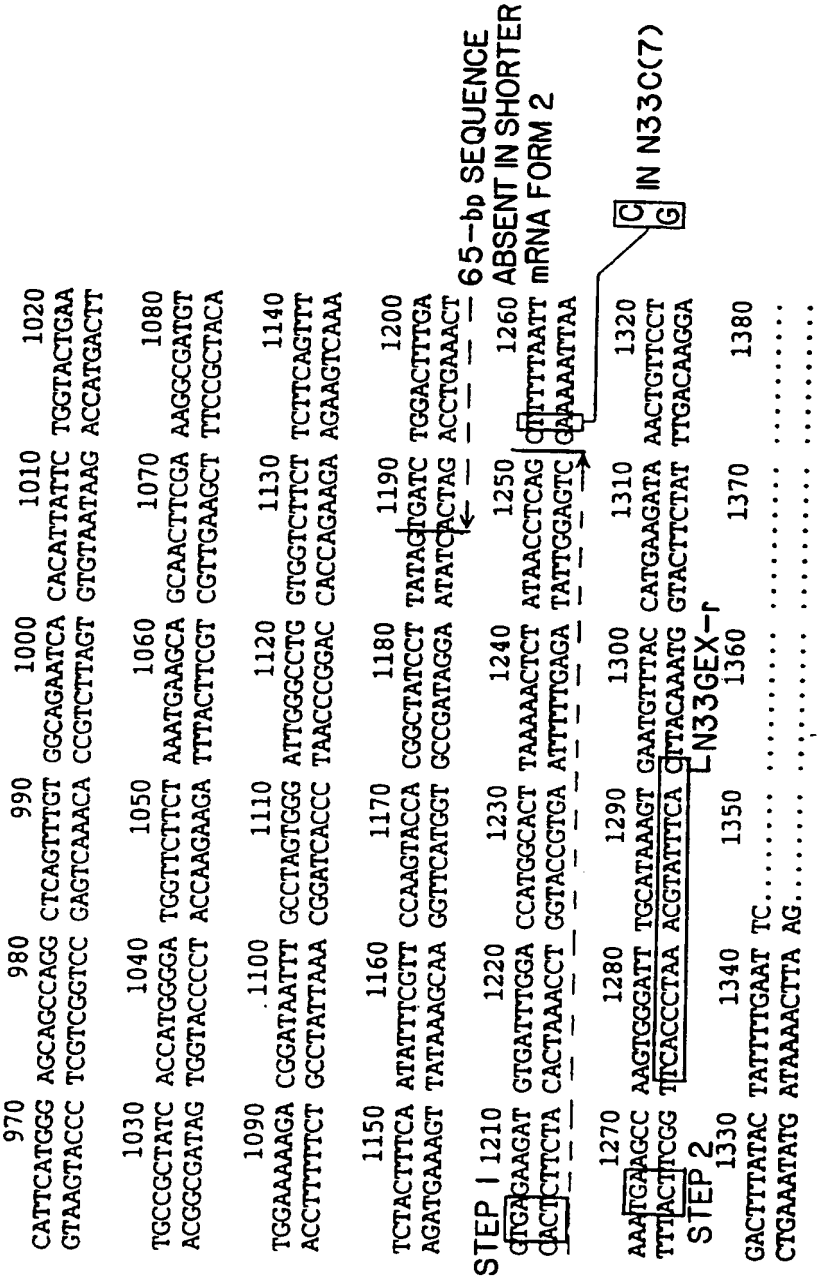
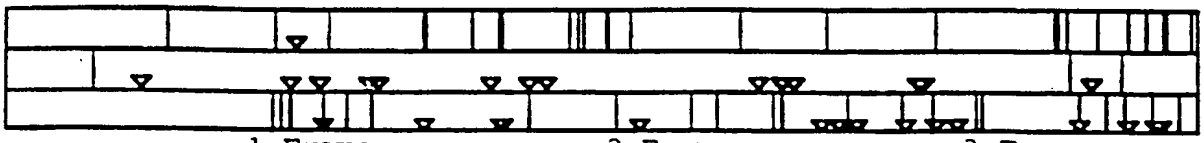

970 980 990 1000 1010 1020
CATTCATGGG AGCAGCCAGG CTCAGTTTGT GGCAGAATCA CACATTATTC TGGTACTGAA
GTAAGTACCC TCGTCGGTCC GAGTCAAACA CCGTCTTAGT GTGTAATAAG ACCATGACTT
1030 1040 1050 1060 1070 1080
TGCCGCTATC ACCATGGGGA TGGTTCTTCT AAATGAAGCA GCAACTTCGA AAGGCGATGT
ACGGCGATAG TGGTACCCCT ACCAAGAAGA TTTACTTCGT CGTTGAAGCT TTCCGCTACA
1090 1100 1110 1120 1130 1140
TGGAAAAAGA CGGATAATTT GCCTAGTGGG ATTGGGCCTG GTGGTCTTCT TCTTCAGTTT
ACCTTTTTCT GCCTATTAAA CGGATCACCC TAACCCGGAC CACCAGAAGA AGAAGTCAAA
1150 1160 1170 1180 1190 1200
TCTACTTTCA ATATTTCGTT CCAAGTACCA CGGCTATCCT TATAGTGATC TGGACTTTGA
AGATGAAAGT TATAAAGCAA GGTTCATGGT GCCGATAGGA ATATCACTAG ACCTGAAACT
65-bp SEQUENCE ABSENT IN SHORTER mRNA FORM 2
STEP 1 1210 1220 1230 1240 1250 1260
GTGAGAAGAT GTGATTTGGA CCATGGCACT TAAAAACTCT ATAACCTCAG TTTTTAATT
CACTCTTCTA CACTAAACCT GGTACCGTGA ATTTTTGAGA TATTGGAGTC AAAAATTAA
1270 1280 1290 1300 1310 1320
AAATGAAGCC AAGTGGGATT TGCATAAAGT GAATGTTTAC CATGAAGATA AACTGTTCCT
TTTACTTCGG TTCACCCTAA ACGTATTTCA GTACAAATG GTACTTCTAT TTGACAAGGA
STEP 2 N33GEX-r
1330 1340 1350 1360 1370 1380
GACTTTATAC TATTTTGAAT TC..... ....
CTGAAATATG ATAAAACTTA AG..... ....
C/G IN N33C(7)

FIG.10B



	1 Frame		2 Frame		3 Frame	
	Init.	Term.	Init.	Term.	Init.	Term.
1	5'	187	5'	104	5'	303
2	5'	307	158	1202	5'	315
3	331	367	326	1202	5'	324
4	331	370	359	1202	5'	360
5	331	472	413	1202	363	387
6	331	475	422	1202	363	414
7	331	526	545	1202	474	591
8	331	556	590	1202	555	591
9	331	559	611	1202	558	591
10	331	637	854	1202	558	690
11	331	646	881	1202	717	777
12	331	655	896	1202	717	807
13	331	682	1034	1202	717	870
14	331	706	1040	1202	717	882
15	331	835	1223	1256	924	954
16	331	931	1223	3'	945	954
17	331	1054			966	1017
18	331	1183			1020	1050
19	331	1186			1053	1095
20	331	1198			1077	1095
21	331	1231			1077	1104
22	331	1264			1209	1212
23	331	1285			1209	1242
24	331	1303			1209	1260
25	331	1309			1263	1290
26	331	1336			1293	1320
27	331	3'			1302	1320
28					1302	3'

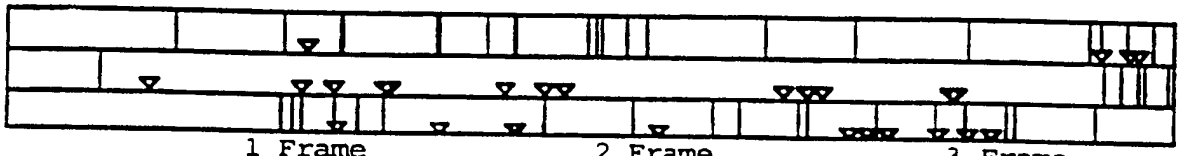
FIG. 11

```
                166         175         184         193         202         211
5'  ATG GGG GCC CGG GGC GCT CCT TCA CGC CGT AGG CAA GCG GGG CGG CGG CTG CGG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Met Gly Ala Arg Gly Ala Pro Ser Arg Arg Arg Gln Ala Gly Arg Arg Leu Arg

                220         229         238         247         256         265
    TAC CTG CCC ACC GGG AGC TTT CCC TTC CTT CTC CTG CTG CTG CTG CTC TGC ATC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Tyr Leu Pro Thr Gly Ser Phe Pro Phe Leu Leu Leu Leu Leu Leu Leu Cys Ile

                274         283         292         301         310         319
    CAG CTC GGG GGA GGA CAG AAG AAA AAG GAG AAT CTT TTA GCT GAA AAA GTA GAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Gln Leu Gly Gly Gly Gln Lys Lys Lys Glu Asn Leu Leu Ala Glu Lys Val Glu

                328         337         346         355         364         373
    CAG CTG ATG GAA TGG AGT TCC AGA CGC TCA ATC TTC CGA ATG AAT GGT GAT AAA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Gln Leu Met Glu Trp Ser Ser Arg Arg Ser Ile Phe Arg Met Asn Gly Asp Lys

                382         391         400         409         418         427
    TTC CGA AAA TTT ATA AAG GCA CCA CCT CGA AAC TAT TCC ATG ATT GTT ATG TTC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Phe Arg Lys Phe Ile Lys Ala Pro Pro Arg Asn Tyr Ser Met Ile Val Met Phe

                436         445         454         463         472         481
    ACT GCT CTT CAG CCT CAG CGG CAG TGT TCT GTG TGC AGG CAA GCT AAT GAA GAA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Thr Ala Leu Gln Pro Gln Arg Gln Cys Ser Val Cys Arg Gln Ala Asn Glu Glu

                490         499         508         517         526         535
    TAT CAA ATA CTG GCG AAC TCC TGG CGC TAT TCA TCT GCT TTT TGT AAC AAG CTC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Tyr Gln Ile Leu Ala Asn Ser Trp Arg Tyr Ser Ser Ala Phe Cys Asn Lys Leu

                544         553         562         571         580         589
    TTC TTC AGT ATG GTG GAC TAT GAT GAG GGG ACA GAC GTT TTT CAG CAG CTC AAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Phe Phe Ser Met Val Asp Tyr Asp Glu Gly Thr Asp Val Phe Gln Gln Leu Asn

                598         607         616         625         634         643
    ATG AAC TCT GCT CCT ACA TTC ATG CAT TTT CCT CCA AAA GGC AGA CCT AAG AGA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Met Asn Ser Ala Pro Thr Phe Met His Phe Pro Pro Lys Gly Arg Pro Lys Arg

                652         661         670         679         688         697
    GCT GAT ACT TTT GAC CTC CAA AGA ATT GGA TTT GCA GCT GAG CAA CTA GCA AAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ala Asp Thr Phe Asp Leu Gln Arg Ile Gly Phe Ala Ala Glu Gln Leu Ala Lys

                706         715         724         733         742         751
    TGG ATT GCT GAC AGA ACG GAT GTT CAT ATT CGG GTT TTC AGA CCA CCC AAC TAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Trp Ile Ala Asp Arg Thr Asp Val His Ile Arg Val Phe Arg Pro Pro Asn Tyr

                760         769         778         787         796         805
    TCT GGT ACC ATT GCT TTG GCC CTG TTA GTG TCG CTT GTT GGA GGT TTG CTT TAT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ser Gly Thr Ile Ala Leu Ala Leu Leu Val Ser Leu Val Gly Gly Leu Leu Tyr
```

FIG. 12A

```
        814         823         832         841         850         859
TTG AGA AGG AAC AAC TTG GAG TTC ATC TAT AAC AAG ACT GGT TGG GCC ATG GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Leu Arg Arg Asn Asn Leu Glu Phe Ile Tyr Asn Lys Thr Gly Trp Ala Met Val

        868         877         886         895         904         913
TCT CTG TGT ATA GTC TTT GCT ATG ACT TCT GGC CAG ATG TGG AAC CAT ATC CGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ser Leu Cys Ile Val Phe Ala Met Thr Ser Gly Gln Met Trp Asn His Ile Arg

        922         931         940         949         958         967
GGA CCT CCA TAT GCT CAT AAG AAC CCA CAC AAT GGA CAA GTG AGC TAC ATT CAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Gly Pro Pro Tyr Ala His Lys Asn Pro His Asn Gly Gln Val Ser Tyr Ile His

        976         985         994        1003        1012        1021
GGG AGC AGC CAG GCT CAG TTT GTG GCA GAA TCA CAC ATT ATT CTG GTA CTG AAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Gly Ser Ser Gln Ala Gln Phe Val Ala Glu Ser His Ile Ile Leu Val Leu Asn

       1030        1039        1048        1057        1066        1075
GCC GCT ATC ACC ATG GGG ATG GTT CTT CTA AAT GAA GCA GCA ACT TCG AAA GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ala Ala Ile Thr Met Gly Met Val Leu Leu Asn Glu Ala Ala Thr Ser Lys Gly

       1084        1093        1102        1111        1120        1129
GAT GTT GGA AAA AGA CGG ATA ATT TGC CTA GTG GGA TTG GGC CTG GTG GTC TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Asp Val Gly Lys Arg Arg Ile Ile Cys Leu Val Gly Leu Gly Leu Val Val Phe

       1138        1147        1156        1165        1174        1183
TTC TTC AGT TTT CTA CTT TCA ATA TTT CGT TCC AAG TAC CAC GGC TAT CCT TAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Phe Phe Ser Phe Leu Leu Ser Ile Phe Arg Ser Lys Tyr His Gly Tyr Pro Tyr

       1192        1201
AGT GAT CTG GAC TTT GAG TGA 3'
--- --- --- --- --- --- ---
Ser Asp Leu Asp Phe Glu ***
```

FIG. 12B



	1 Frame		2 Frame		3 Frame	
	Init.	Term.	Init.	Term.	Init.	Term.
1	5'	187	5'	104	5'	303
2	5'	307	158	1199	5'	315
3	331	367	326	1199	5'	324
4	331	370	359	1199	5'	360
5	331	472	413	1199	363	387
6	331	475	422	1199	363	414
7	331	526	545	1199	474	591
8	331	556	590	1199	555	591
9	331	559	611	1199	558	591
10	331	637	854	1199	558	690
11	331	646	881	1199	717	777
12	331	655	896	1199	717	807
13	331	682	1034	1199	717	870
14	331	706	1040	1199	717	882
15	331	835	1040	1220	924	954
16	331	931	1040	1238	945	954
17	331	1054	1040	1244	966	1017
18	331	1183	1040	1271	1020	1050
19	331	1195	1040	3'	1053	1095
20	1198	1225			1077	1095
21	1228	1255			1077	1104
22	1237	1255			1077	1191
23	1237	3'			1077	3'

FIG. 13

```
          166        175        184        193        202        211
5'  ATG GGG GCC CGG GGC GCT CCT TCA CGC CGT AGG CAA GCG GGG CGG CGG CTG CGG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Met Gly Ala Arg Gly Ala Pro Ser Arg Arg Arg Gln Ala Gly Arg Arg Leu Arg

          220        229        238        247        256        265
    TAC CTG CCC ACC GGG AGC TTT CCC TTC CTT CTC CTG CTG CTG CTG CTC TGC ATC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Tyr Leu Pro Thr Gly Ser Phe Pro Phe Leu Leu Leu Leu Leu Leu Cys Ile

          274        283        292        301        310        319
    CAG CTC GGG GGA GGA CAG AAG AAA AAG GAG AAT CTT TTA GCT GAA AAA GTA GAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Gln Leu Gly Gly Gly Gln Lys Lys Lys Glu Asn Leu Leu Ala Glu Lys Val Glu

          328        337        346        355        364        373
    CAG CTG ATG GAA TGG AGT TCC AGA CGC TCA ATC TTC CGA ATG AAT GGT GAT AAA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Gln Leu Met Glu Trp Ser Ser Arg Arg Ser Ile Phe Arg Met Asn Gly Asp Lys

          382        391        400        409        418        427
    TTC CGA AAA TTT ATA AAG GCA CCA CCT CGA AAC TAT TCC ATG ATT GTT ATG TTC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Phe Arg Lys Phe Ile Lys Ala Pro Pro Arg Asn Tyr Ser Met Ile Val Met Phe

          436        445        454        463        472        481
    ACT GCT CTT CAG CCT CAG CGG CAG TGT TCT GTG TGC AGG CAA GCT AAT GAA GAA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Thr Ala Leu Gln Pro Gln Arg Gln Cys Ser Val Cys Arg Gln Ala Asn Glu Glu

          490        499        508        517        526        535
    TAT CAA ATA CTG GCG AAC TCC TGG CGC TAT TCA TCT GCT TTT TGT AAC AAG CTC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Tyr Gln Ile Leu Ala Asn Ser Trp Arg Tyr Ser Ser Ala Phe Cys Asn Lys Leu

          544        553        562        571        580        589
    TTC TTC AGT ATG GTG GAC TAT GAT GAG GGG ACA GAC GTT TTT CAG CAG CTC AAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Phe Phe Ser Met Val Asp Tyr Asp Glu Gly Thr Asp Val Phe Gln Gln Leu Asn

          598        607        616        625        634        643
    ATG AAC TCT GCT CCT ACA TTC ATG CAT TTT CCT CCA AAA GGC AGA CCT AAG AGA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Met Asn Ser Ala Pro Thr Phe Met His Phe Pro Pro Lys Gly Arg Pro Lys Arg

          652        661        670        679        688        697
    GCT GAT ACT TTT GAC CTC CAA AGA ATT GGA TTT GCA GCT GAG CAA CTA GCA AAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ala Asp Thr Phe Asp Leu Gln Arg Ile Gly Phe Ala Ala Glu Gln Leu Ala Lys

          706        715        724        733        742        751
    TGG ATT GCT GAC AGA ACG GAT GTT CAT ATT CGG GTT TTC AGA CCA CCC AAC TAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Trp Ile Ala Asp Arg Thr Asp Val His Ile Arg Val Phe Arg Pro Pro Asn Tyr

          760        769        778        787        796        805
    TCT GGT ACC ATT GCT TTG GCC CTG TTA GTG TCG CTT GTT GGA GGT TTG CTT TAT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ser Gly Thr Ile Ala Leu Ala Leu Leu Val Ser Leu Val Gly Gly Leu Leu Tyr
```

FIG. 14A

```
          814         823         832         841         850         859
TTG AGA AGG AAC AAC TTG GAG TTC ATC TAT AAC AAG ACT GGT TGG GCC ATG GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Leu Arg Arg Asn Asn Leu Glu Phe Ile Tyr Asn Lys Thr Gly Trp Ala Met Val

          868         877         886         895         904         913
TCT CTG TGT ATA GTC TTT GCT ATG ACT TCT GGC CAG ATG TGG AAC CAT ATC CGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ser Leu Cys Ile Val Phe Ala Met Thr Ser Gly Gln Met Trp Asn His Ile Arg

          922         931         940         949         958         967
GGA CCT CCA TAT GCT CAT AAG AAC CCA CAC AAT GGA CAA GTG AGC TAC ATT CAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Gly Pro Pro Tyr Ala His Lys Asn Pro His Asn Gly Gln Val Ser Tyr Ile His

          976         985         994        1003        1012        1021
GGG AGC AGC CAG GCT CAG TTT GTG GCA GAA TCA CAC ATT ATT CTG GTA CTG AAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Gly Ser Ser Gln Ala Gln Phe Val Ala Glu Ser His Ile Ile Leu Val Leu Asn

         1030        1039        1048        1057        1066        1075
GCC GCT ATC ACC ATG GGG ATG GTT CTT CTA AAT GAA GCA GCA ACT TCG AAA GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ala Ala Ile Thr Met Gly Met Val Leu Leu Asn Glu Ala Ala Thr Ser Lys Gly

         1084        1093        1102        1111        1120        1129
GAT GTT GGA AAA AGA CGG ATA ATT TGC CTA GTG GGA TTG GGC CTG GTG GTC TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Asp Val Gly Lys Arg Arg Ile Ile Cys Leu Val Gly Leu Gly Leu Val Val Phe

         1138        1147        1156        1165        1174        1183
TTC TTC AGT TTT CTA CTT TCA ATA TTT CGT TCC AAG TAC CAC GGC TAT CCT TAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Phe Phe Ser Phe Leu Leu Ser Ile Phe Arg Ser Lys Tyr His Gly Tyr Pro Tyr

         1192        1201
AGC TTT TTA ATT AAA TGA 3'
--- --- --- --- --- ---
Ser Phe Leu Ile Lys ***
```

FIG. 14B

```
                        10         20         30         40         50
N33 Form 1            1 MGARGAPSRR RQAGRRLRYL PTGSFPFLLL LLLLCIQLGG GQKKKENLLA      50
N33 Form 2            1 MGARGAPSRR RQAGRRLRYL PTGSFPFLLL LLLLCIQLGG GQKKKENLLA      50
ZK686.3 cDNA          1 ---------- ---------- ---------- -----MLLAV YESAQQQTLE      50

                        60         70         80         90        100
N33 Form 1           51 EKVEQLMEWS SRRSIFRMNG DKFRKFIKAP PRNYSMIVMF TALQPQRQCS     100
N33 Form 2           51 EKVEQLMEWS SRRSIFRMNG DKFRKFIKAP PRNYSMIVMF TALQPQRQCS     100
ZK686.3 cDNA         51 DKVQNLVDLT SRQSIVKFNM DKWKTLVRMQ PRNYSMIVMF TALSPGVQCP     100

                       110        120        130        140        150
N33 Form 1          101 VCRQANEEYQ ILANSWRY-S SAFCN-KLFF SMVDYDEGTD VFQQLNMNSA     150
N33 Form 2          101 VCRQANEEYQ ILANSWRY-S SAFCN-KLFF SMVDYDEGTD VFQQLNMNSA     150
ZK686.3 cDNA        101 ICKPAYDEFM IVANSHRYTS SEGDRRKVFF GIVDYEDAPQ IFQQMNLNTA     150

                       160        170        180        190        200
N33 Form 1          151 PTFMHFPPK- GRPKRADTFD LQRIGFAAEQ LAKWIADRTD VHIRVFRPPN     200
N33 Form 2          151 PTFMHFPPK- GRPKRADTFD LQRIGFAAEQ LAKWIADRTD VHIRVFRPPN     200
ZK686.3 cDNA        151 PILYHFGPKL GAKKRPEQMD FQRQGFDADA IGRFVADQTE VHVRVIRPPN     200

                       210        220        230        240        250
N33 Form 1          201 YSGTIALALL VSLVGGLLYL RRNNLEFIYN KTGWAMVSLC IVFAMTSGQM     250
N33 Form 2          201 YSGTIALALL VSLVGGLLYL RRNNLEFIYN KTGWAMVSLC IVFAMTSGQM     250
ZK686.3 cDNA        201 YTAPVVIALF VALLLGMLYM KRNSLDFLFN RTVWGFVCLA ITFIFMSGQM     250

                       260        270        280        290        300
N33 Form 1          251 WNHIRGPPYA HKNPHNGQVS YIHGSSQAQF VAESHIILVL NAAITMGMVL     300
N33 Form 2          251 WNHIRGPPYA HKNPHNGQVS YIHGSSQAQF VAESHIILVL NAAITMGMVL     300
ZK686.3 cDNA        251 WNHIRGPPFM ITNPNTKEPS FIHGSTQFQL IAETYIVGLL YALIAIGFIC     300

                       310        320        330        340        350
N33 Form 1          301 LNEAATSKGD VGKRRII--- ---------- ---CLVGLGL VVFFFSFLLS     350
N33 Form 2          301 LNEAATSKGD VGKRRII--- ---------- ---CLVGLGL VVFFFSFLLS     350
ZK686.3 cDNA        301 VNEAADQSNS KDRKNAGKKL NPLSLLNIPT NTLAIAGLVC ICVFFSFLLS     350

                       360        370        380        390        400
N33 Form 1          351 IFRSKYHGYP YSDLDFE*.. .......... .......... ..........     400
N33 Form 2          351 IFRSKYHGYP YSFLIK*... .......... .......... ..........     400
ZK686.3 cDNA        351 VFRSKYRGYP YSFLFA*... .......... .......... ..........     400
```

FIG. 15

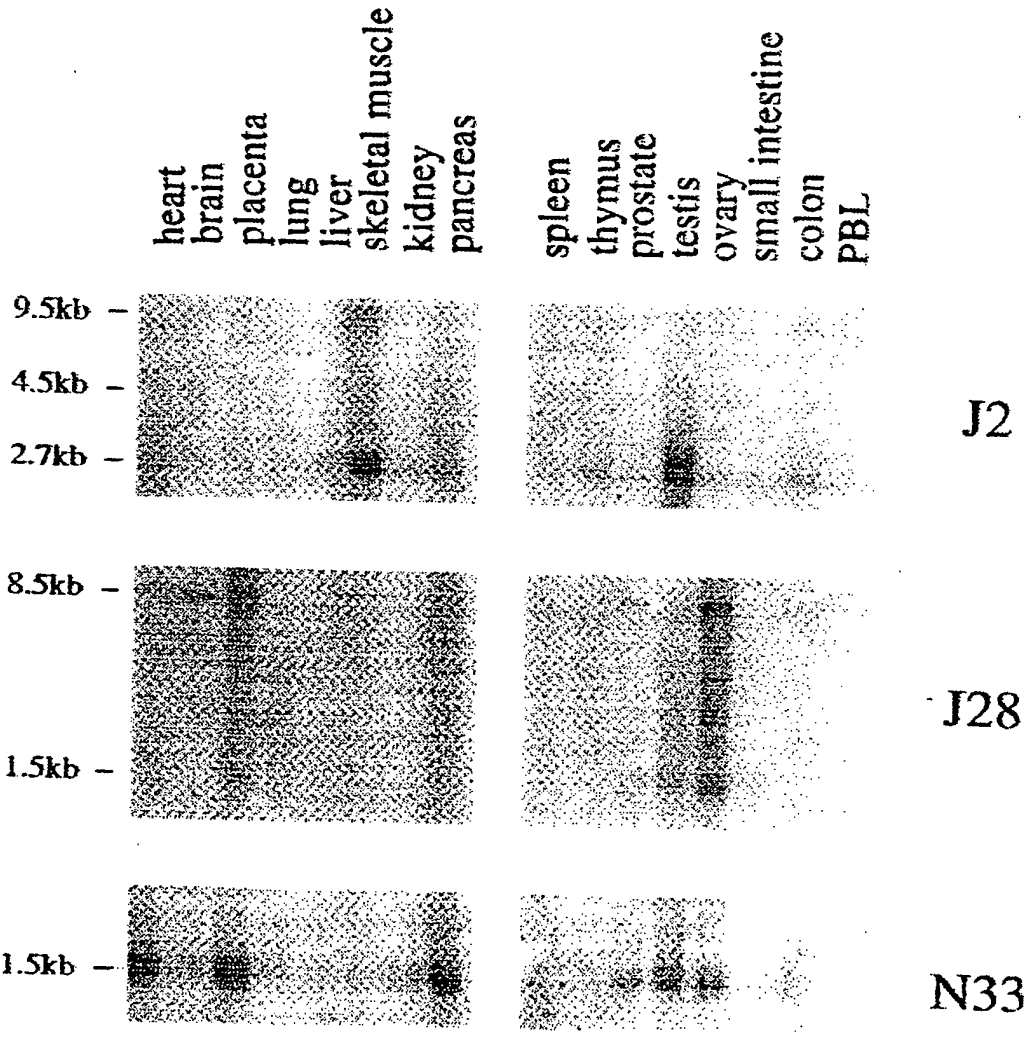

heart
brain
placenta
lung
liver
skeletal muscle
kidney
pancreas
spleen
thymus
prostate
testis
ovary
small intestine
colon
PBL
9.5kb
4.5kb
2.7kb
J2
8.5kb
1.5kb
J28
1.5kb
N33

FIG.16

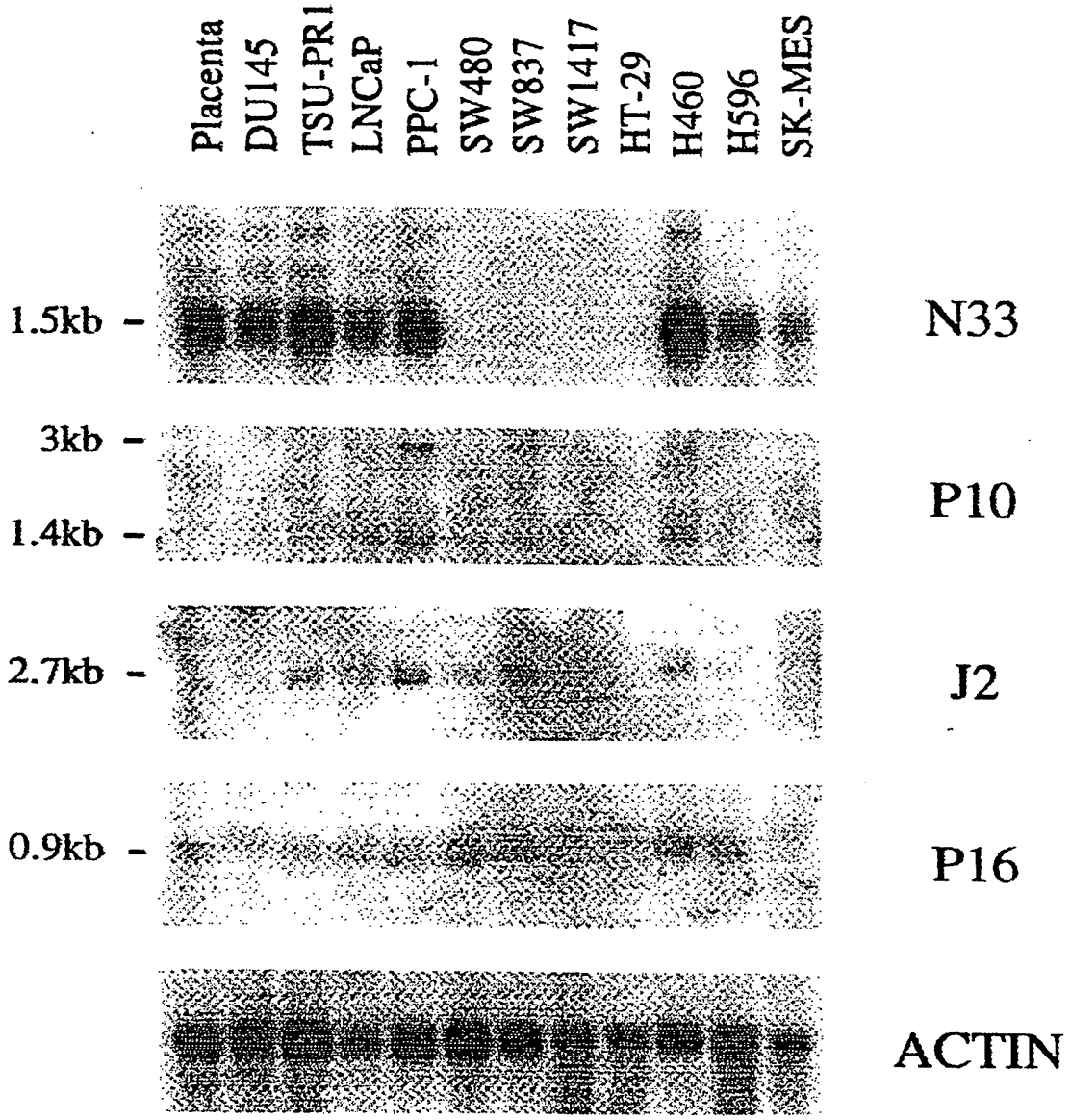

Placenta
DU145
TSU-PR1
LNCaP
PPC-1
SW480
SW837
SW1417
HT-29
H460
H596
SK-MES
1.5kb
3kb
1.4kb
2.7kb
0.9kb
N33
P10
J2
P16
ACTIN

FIG.17A

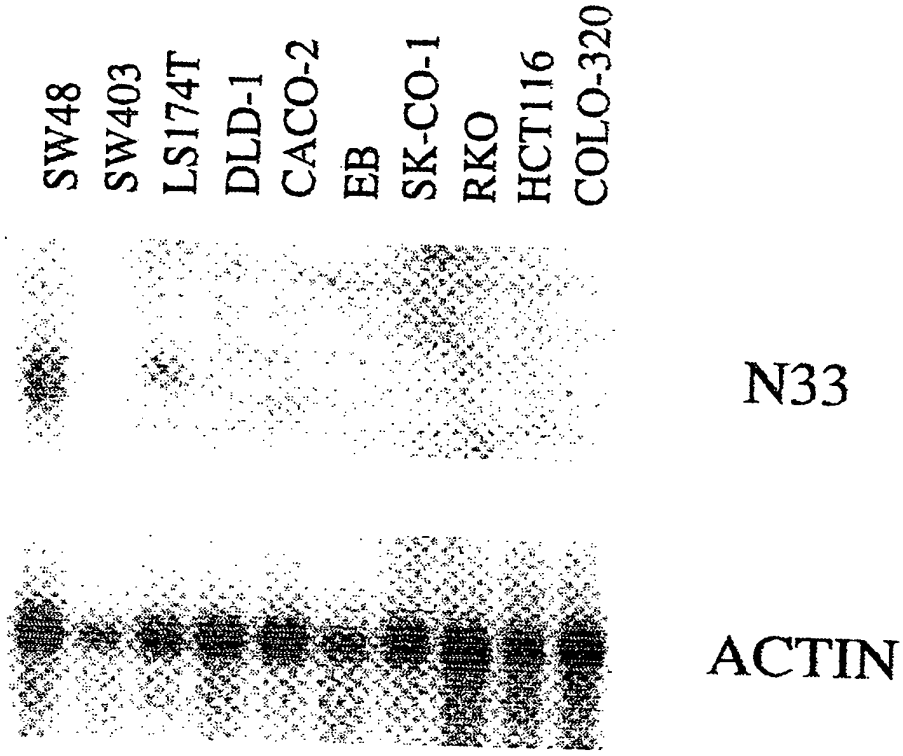


FIG.17B

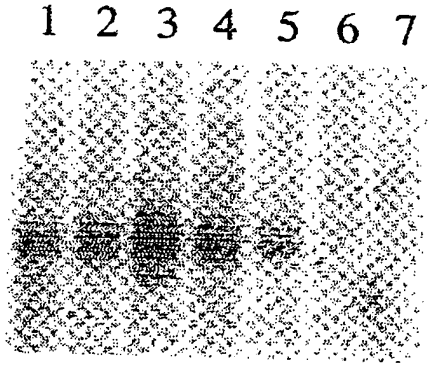


FIG.18

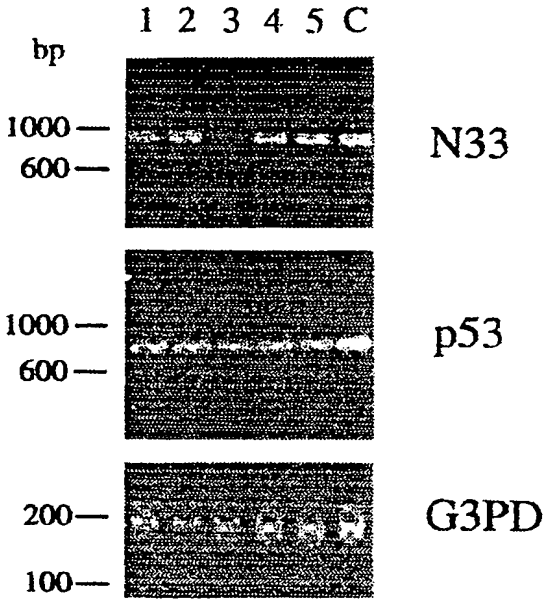


FIG. 19A

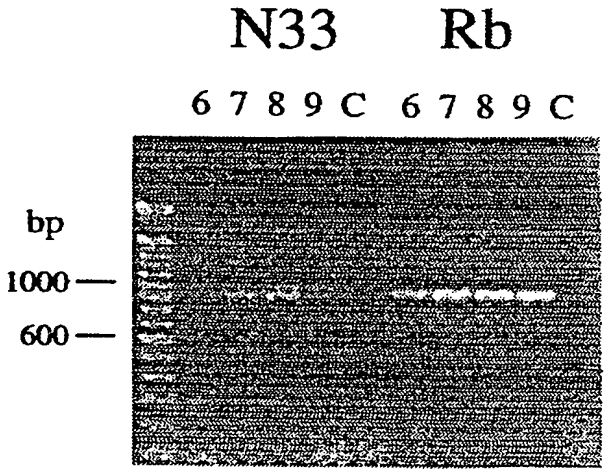


FIG. 19B

```
        10         20         30         40         50         60
MGARGAPSRR RQAGRRLRYL PTGSFPFLLL LLLLCIQLGG GQKKKENLLA EKVEQLMEWS

        70         80         90        100        110        120
SRRSIFRMNG DKFRKFIKAP PRNYSMIVMF TALQPQRQCS VCRQANEEYQ ILANSWRYSS

       130        140        150        160        170        180
AFCNKLFFSM VDYDEGTDVF QQLNMNSAPT FMHFPPKGRP KRADTFDLQR IGFAAEQLAK

       190        200        210        220        230        240
WIADRTDVHI RVFRPPNYSG TIALALLVSL VGGLLYLRRN NLEFIYNKTG WAMVSLCIVF

       250        260        270        280        290        300
AMTSGQMWNH IRGPPYAHKN PHNGQVSYIH GSSQAQFVAE SHIILVLNAA ITMGMVLLNE

       310        320        330        340        350        360
AATSKGDVGK RRIICLVGLG LVVFFFSFLL SIFRSKYHGY PYSDLDFE*. ..........
```

FIG.20

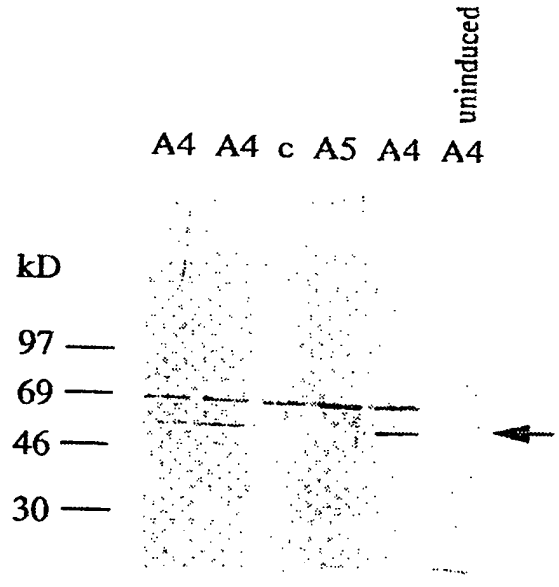


FIG.21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06593

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 70.1, 70.3, 71.1, 71.2, 240.1, 320.1; 530/324, 350; 536/23.5, 24.3, 24.31; 930/10

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, MASPAR, GENBANK 89, GENBANK-NEW 6, EMBL-NEW 6, UEMBL 43 89, N-GENESEQ 18, SWISS-PROT 31, PIR 44, A-GENESEQ 18
search terms: chromosom##, eight#, 8#, suppress###

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	American Journal of Pathology, Vol. 144, No. 1, issued January 1994, Chang et al, "Deletion Mapping of Chromosome 8p in Colorectal Carcinoma and Dysplasia Arising in Ulcerative Colitis, Prostatic Carcinoma, and Malignant Fibrous Histiocytomas", pages 1-6, see entire document.	1-21,30,31
A	Cancer Research, Vol. 54, No. 9, issued 01 May, 1994, Ichikawa et al, "Suppression of Metastasis of Rat Prostatic Cancer by Introducing Human Chromosome 8", pages 2299-2302, see entire document.	1-21,30,31

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
29 AUGUST 1995

Date of mailing of the international search report
15SEP1995

Name and mailing address of the ISA/US
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Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06593

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-21, 30, and 31

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06593

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 21/04; C07K 1/00, 14/00; C12N 5/00, 15/00, 15/09, 15/11, 15/12, 15/63; C12P 21/04, 21/06

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 70.1, 70.3, 71.1, 71.2, 240.1, 320.1; 530/324, 350; 536/23.5, 24.3, 24.31; 930/10

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.
Group I, claim(s) 1-21, 30, and 31, are drawn to a single inventive concept of nucleic acids which encode for a tumor suppressor protein, probes for the nucleic acids, the suppressor protein itself, and method of making the protein.
Group II, claim(s) 25-29, drawn to an antibody and method of using an antibody.
Group III, claim(s) 22-23, drawn to gene therapy.
Group IV, claim(s) 24, drawn to therapy utilizing the protein.
The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Group II is drawn to an antibody and a method of using the antibody which is a materially and functionally different and distinct protein than a tumor suppressor protein and a tumor suppressor protein is not required or used in the method of using the antibody. Furthermore, the antibody is not encoded by the special technical feature of nucleic acids that encode for a tumor suppressor protein.
Group III is drawn to a method of gene therapy that includes in vivo methods and materials that does not share the special technical feature of nucleic acids that encode for a tumor suppressor protein.
Group IV is drawn to a method of therapy utilizing a tumor suppressor protein that includes in vivo methods and materials that does not share the special technical feature of nucleic acids that encode for a tumor suppressor protein.
The nucleic acids, probes for the nucleic acids, and a tumor suppressor protein they encode of Group I, and the antibody of Group II have materially different structural and functional properties, each from the other. Thus the special technical features which identify each also distinguish each from the other.
Group I's method of making a tumor suppressor protein, Group II's method of using an antibody, Group III's method of gene therapy, and Group IV's method of tumor suppressor protein therapy each use process steps and compositions that are materially different from the others and are unique to the group. Thus the special technical features that define each method distinguish the methods each from the other.
Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.